Dhiraj Kumar
Chengliang Gong *Editors*

Trends in Insect Molecular Biology and Biotechnology

Springer

Trends in Insect Molecular Biology and Biotechnology

Dhiraj Kumar • Chengliang Gong
Editors

Trends in Insect Molecular Biology and Biotechnology

Springer

Editors
Dhiraj Kumar
School of Biology and
Basic Medical Sciences
Soochow University
Suzhou
China

Chengliang Gong
School of Biology and
Basic Medical Sciences
Soochow University
Suzhou
China

ISBN 978-3-319-61342-0 ISBN 978-3-319-61343-7 (eBook)
https://doi.org/10.1007/978-3-319-61343-7

Library of Congress Control Number: 2017964663

Printed on acid-free paper

This Springer imprint is published by the registered company Springer International Publishing AG part of Springer Nature
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Preface

Insects are one of the versatile groups of the animal kingdom with a large population and are long since attracting researchers to disclose their molecular biology and use them for the benefit of humankind. Several traditional insects such as silkworms, honey bee, lac, *Drosophila*, termites, etc. are genetically and economically important and are the best invertebrate animal models. In the modern era of genetic engineering, these insects open a new horizon in the molecular biology with a multidisciplinary approach. Additionally, insect-derived products are widely used in biomedical and biotechnology industries. In this book, we made an effort to club together various recent aspects of insect molecular biology, including insect genomics, proteomics, virology, noncoding RNA, nano-biotechnology, recombinant insect products, and their applications in modern research. Therefore, this volume will certainly help academics and scientists to better understand and carry out research on insect genomics, proteomics, and transgenics and their utilization. The first section of this book comprises topics on insect molecular marker-assisted selection in breeding, molecular mechanism of communication, monocyclic aromatic hydrocarbons (MAHs)-induced toxicity, molecular studies of evolution, long noncoding RNA discovery, and pathogen-driven proteomics of various insects. The second part describes the role of viral lytic polysaccharide monooxygenase, antiviral mechanism, and RNAi as a novel tool for crop protection. The third section deals with the application of recombinant insect products and chitinolytic enzymes, the role of insects in forensic science, and genome research on *Cordycep*, including information on nanotechnology application in insect molecular biology. The content of the book will also provide a common platform for the molecular entomologist and biotechnologist to develop novel, significant, and accessible approach for mankind across the world.

Suzhou, China — Dhiraj Kumar
Suzhou, China — Chengliang Gong

Acknowledgements

It is a great privilege to express my humble gratitude to all the contributing authors of this book, who not only provided support but also read, wrote, offered comments, and allowed me to quote their remarks and assisted in the editing, proofreading, and design. I am extremely thankful to my co-editor Prof. Chengliang Gong, Soochow University, China, for his collaboration, contribution, critical review, and suggestions. Without his help and sincere efforts, it was not possible to complete this book.

I feel elevated to express my deep sincere regards and a profound sense of gratitude to Prof. Jose L. Cenis, Spain, Dr. Venkatesh Kumar R., and Dr. Rajesh Kundapur for their encouragement, persistence, and constructive and critical suggestions, throughout the tenure of my book editing. It was their aptness and precision in perception that helped me in completing the book.

There are no words to convey my emotions and gratitude to my respected parents, Shri Jiya Lal and Smt. Rita Devi, for their moral support, love, inspiration, and blessing to make this volume happen. I express my special thanks to Dr. Madhavi, India, who always motivated and helped me to complete this assignment in the form of a book.

Contents

About the Editors



Dr. Dhiraj Kumar completed his post-doctorate at the Molecular Biology Laboratory, School of Biology and Basic Medical Science, Soochow University, China, and selected as a visiting scientist in the prestigious Talented Young Scientist Programme of the Chinese Government. He also worked as an Assistant Professor at the Department of Zoology, Guru Ghasidas Vishwavidyalaya (A Central University), Bilaspur (C.G.), India. Dr. Kumar received a young scientist award and an outstanding paper award for his research contributions in biological science. He has published numerous research papers in renowned international journals including those from the Nature Publishing Group and has filed a patent for his new innovation. Presently Dr. Kumar's research is focused on metagenomics, transgenic technology, and biomedical science.



Dr. Chengliang Gong is currently serving at Soochow University, China, as a Professor and Dean of the Department of Applied Biology, School of Biology and Basic Medical Science. He is also Vice President of the Sericultural Society of Jiangsu Province, China. Prof. Gong has carried out over forty research projects as a PI funded by various research funding organizations of China and has published more than fifty research papers in international journals. He has also obtained ten patents as the first inventor. His major research interests are in the areas of genetic manipulation, molecular biology, biochemistry, and pathology of insects and aquatic animals.

Part I

Insect Genomics and Proteomics

1 Molecular Marker-Assisted Selection Breeding in Silkworm, *Bombyx mori*

Rajendra Mundkur and E. Muniraju

Abstract

Silkworm is the lepidopteran molecular model. Many biotechnologists have constructed large database of DNA sequences and have tried to correlate with the traditional linkage maps. Molecular marker-assisted selection (MAS) has been considered as a means to improve the efficiency, accuracy, and speed of the breeding process. Markers are the specialized breeding tools which aid selection for target genes that are not easily visible morphologically in individuals, minimize the drag around the target gene, and reduce the number of generations required to achieve the required result. In this hope, conventional breeders in all the disciplines of life sciences, including silkworm, are turning into biotechnologists. Like in any field of computer science, before a technology in the field of DNA marker is understood and put into practice, another simpler, more efficient, economical technology emerges. In this context, to keep up the pace with the technology, it has become necessary for the breeder to be familiar with the advancement in the field of DNA-marker technology.

1.1 Introduction

The silkworm, *Bombyx mori*, known to mankind since more than 4700 years is a highly domesticated and well-characterized genetic tool (Tazima 1962) next to the fruit fly, *Drosophila*. After the advent of recombinant DNA technology in breeding, the silkworm has become the lepidopteran molecular model system (Goldsmith

R. Mundkur (✉) • E. Muniraju
Karnataka State Sericulture Research and Development Institute Thalaghattapura, 1098, 26 B Main Road, Jayanagar 9th Block, Bangalore 560 109, Karnataka, India
e-mail: rajendra.mundkur@gmail.com

D. Kumar, C. Gong (eds.), *Trends in Insect Molecular Biology and Biotechnology*, https://doi.org/10.1007/978-3-319-61343-7_1

1995). In Japan, the information about improved races are available (Yokoyama 1973) since as early as 1680. Till and even after the Mendelian era, the breeding was often referred to as an art because a new breed developed based on the experience and ingenuity of the breeder in correlating phenotypic expression with the genetic makeup of the individual. Later, systematic breeding started using the principles of traditional genetics**.** Though the knowledge of genes governing phenotypic expressions was prevailing, there were no tools to have access to the genes. Characterization of the silkworm genome was fast developed because of its importance as lepidopteran model for breeding and genetic studies, for isolating valuable genes and promoters, and for comparative genomics (Goldsmith et al. 2005; Goldsmith 2006). This included molecular linkage maps, BAC libraries, large EST databases, and whole-genome shotgun sequences (Goldsmith 2006). In contrast to the traditional genetics, where individual organisms are used for crossing and genetic studies, modern-day genomic studies involve test tubes, micropipettes, gels, and liquid media. Along with the liquid genetics, the computational biology and bioinformatics provide insight into the genetic molecules and their expression.

The comparative increase in silk yield over 90 years of conventional breeding in Japan (Kuribayashi 1992) (Table 1.1) indicates that the cocoon production/50 dfls has increased by 386.5% and raw silk % by 291.3%.

Thereafter, the increase in quantitative traits has become plateau. Employing biotechnological tools becomes a necessity to achieve marked quantum jump beyond this level. The recent DNA molecular architecture of silkworm, *Bombyx* mori (Gage 1974; Yasukochi 1998; Wu et al. 1999; Wang et al. 2005), is indicated in Table 1.2.

Breeders are contemplating on designing the breeding program using the molecular information. This involves many terminologies and processes routinely used in molecular biotechnology. The terminologies and processes that are related to the DNA marker-assisted selection (MAS) breeding are discussed in this article.

1.2 Quantitative Trait Locus (QTL)

A quantitative trait locus (Beavis et al. 1991) is a region of DNA that is associated with a particular phenotypic trait. These QTLs are often found on different chromosomes. Knowing the number of QTLs that explains variation in the phenotypic trait indicates the genetic architecture of a trait. For example, it may suggest that plant height is controlled by many genes of small effect or by a few genes of large effect.

Another use of QTLs is to identify candidate genes underlying a trait. Once a region of DNA is identified as contributing to a phenotype, it can be sequenced. The DNA sequence of any genes in this region can then be compared to a database of DNA for genes whose function is already known. Advancement in quantitative genetics and work on QTL facilitated linking of certain markers to the gene of focus, thereby increasing the accuracy of breeding values.

In a recent development, classical QTL analyses are combined with gene expression profiling, i.e., by DNA microarrays. Such expression QTLs (e-QTLs) describes *cis*- and *trans*-controlling elements for the expression of often disease-associated

Table 1.1 Increase in quantitative productivity over 90 years in Japan through conventional breeding

Character	1890–1904	1987–1991	% increase
Cocoon yield/50 dfls (kg)	8.9	34.40	386.5
Raw silk (%)	7.3	21.27	291.3

Table 1.2 DNA molecular architecture of *Bombyx mori* (Wang et al. 2005; Gage 1974; Yasukochi 1998; Wu et al. 1999)

Description	
Haploid genome size	~530 mb
Linkage groups	28
Linkage map distance	1000 cM
Number of cloned genes	>20
Silk gland genes	1874
Genetic markers	1018
Average interval between markers	~2 cM (=~500 kb)
No. of RFLP markers	61
No. of RAPD markers	168
No. of RAPD primers	140
Independent cDNA BAC libraries	>11,000
cDNA libraries	209
EST	64,038
Scaffolds	23,156
Contigs	66,482
SSRs	601,225
Genes predicted by BGI gene finder	21,302
Homologous genes from other lepidopterans	521

genes. Knowledge of QTLs therefore becomes essential for marker-assisted selection (MAS) in breeding program (Nagaraju and Goldsmith 2002).

1.3 Reverse Genetics

Reverse genetics is an approach to discover the function of a gene that proceeds in the opposite direction of so-called forward genetics or classical genetics. While forward genetics seeks to find the genetic basis of a phenotype or trait, reverse genetics seeks to find the possible phenotypes that may derive from a specific genetic sequence enumerated during DNA sequencing. Due to the modern techniques of DNA sequencing, vast amounts of genomic sequence data become available, and many genetic sequences are discovered in advance of other information. Reverse genetics attempts to connect a given genetic sequence with specific effects on the organism. This is the reversal of central dogma where the information flows from protein to mRNA to cDNA.

1.4 Genome Annotation

Genome annotation (Ghedin et al. 2004) is the process of marking the genes and other biological features in a DNA sequence by genome annotation software system (White 1995). The software system is used to find the genes (places in the DNA sequence that encode a protein), the transfer RNA, and other features and to make initial assignments of function to those genes. The advanced genome annotation software systems work similarly, but the programs available for analysis of genomic DNA are constantly changing and improving.

1.5 Extraction of DNA

Both plasmids and chromosomal DNA molecules can be isolated from cells. In both cases, the cell membrane is solubilized by the application of detergent. The resultant lysate is then enzyme/heat treated to remove various contaminants from the desired DNA. The DNA molecules are collected by precipitating them into ethanol.

1.6 Cutting DNA into Small Fragments

The restriction enzyme, found in bacterial cells, functions to protect those cells from infection by bacteriophage particles. They carry out this function by cleaving the invading phage DNA. DNA of the host cell remains protected by attaching methyl group to some of its bases. The restriction enzymes bind to specific sequences of bases known as recognition sequences. Each enzyme has a single specific recognition sequence, e.g., EcoRI (pronounced as eco-r-one) (restriction enzyme I of *E. coli*.) restriction enzyme cuts DNA whenever the base sequence **GAATTC** is found.

Once the enzyme has bound to the specific base sequence, it cleaves the DNA backbone, thus breaking the molecule into fragments. The most useful enzymes, known as Type II restriction endonucleases, cleave the DNA molecule at a predictable site within the recognition sequence itself.

Each restriction enzyme, while cleaving, leaves two characteristic ends (terminals) on the fragments: (1) blunt ends, in which there is no overhanging single-strand tail, and (2) staggered ends, in which a single-strand tail overhangs, either in 3′ or 5′ direction.

The staggered ends sometimes complement one another, in which case the staggered ends tend to hydrogen bond to one another and are thus called “sticky ends.” Sticky ends make it possible to join two DNA fragments together, regardless of the source of DNA. The enzyme DNA ligase functions to complete the sugar-phosphate backbone between the newly joined (ligated) fragments. When the fragments are hybridized from at least two different sources, the resultant ligated molecule is called recombinant DNA (rDNA).

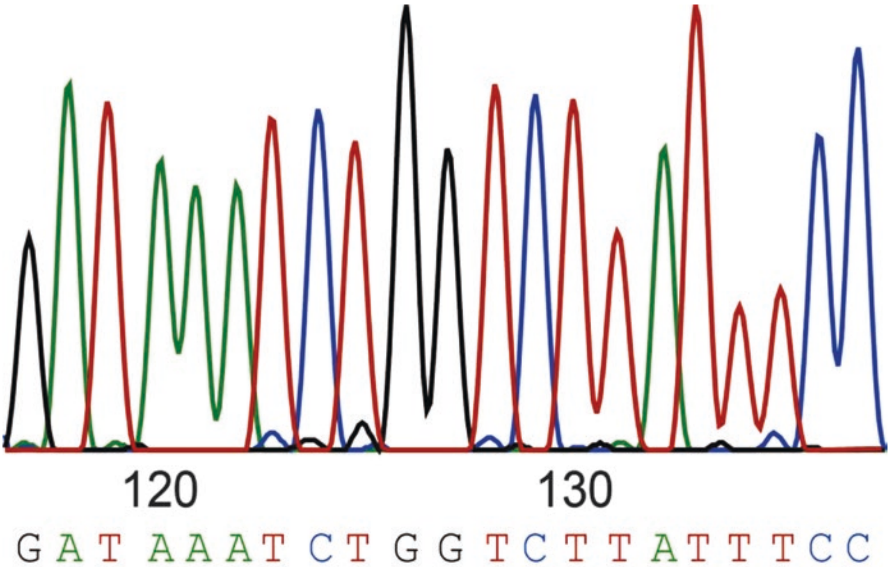


Fig. 1.1 Electropherogram printout from automated sequencer showing part of a DNA sequence (Source: Wikimedia 2003)

1.7 DNA Sequencing

The base sequence of DNA molecules can be determined using a variety of techniques (Fig. 1.1).

1.7.1 The Maxam-Gilbert technique

The Maxam-Gilbert technique (Maxam and Gilbert 1977) depends on the selective degradation of specific bases within the molecule to be sequenced. The degradation results in the production of large population of DNA fragments which are separated by using PAGE. The resulting band pattern determined by autoradiography is used to determine the base sequence. Based on this protocol, more simple and advanced methods have been developed.

1.7.2 The Sanger dideoxy DNA sequencing technique

The Sanger dideoxy DNA sequencing technique (Sanger et al. 1977) relies on the interruption of DNA synthesis. By adding a carefully calculated amount of modified dideoxynucleotides to the reaction mixture of DNA subunits, it is possible to halt DNA chain elongation at various base sites, resulting in a wide variety of fragments of DNA. This approach is also known as "dye-primer sequencing."

1.7.3 Pyrosequencing

Pyrosequencing is a method of DNA sequencing based on the "sequencing by synthesis" principle developed initially by Pal Nyren and coworkers (1985–1990) (Babak et al. 2004). The method is based on a chemiluminescent enzymatic reaction, which is triggered when a molecular recognition event occurs. Essentially, the method allows sequencing of a single strand of DNA by synthesizing the complementary strand along it. Each time a nucleotide, A, C, G, or T, is incorporated into the growing chain, a cascade of enzymatic reactions is triggered which results in a light signal. It is a method primarily used for sequencing of short stretches of DNA, SNP detection, and methylation analysis. Such analyses are crucial for biological research, genetics, and some medical and forensic applications. Pyrosequencing is fully automated, reliable, and accurate, and large numbers of samples can be analyzed in a short time. Pyrosequencing methods have been pursued to reduce costs relative to other automated sequencing methods.

1.7.4 Shotgun Sequencing

Shotgun sequencing (Anderson 1981) is used for sequencing long DNA strands. Since the chain termination method of DNA sequencing can only be used for fairly short strands, it is necessary to divide longer sequences up and then assemble the results to give the overall sequence. In chromosome walking, this division is done by progressing through the entire strand, piece by piece; shotgun sequencing uses a faster but more complex process to assemble random pieces of the sequence.

In shotgun sequencing, DNA is broken up randomly into numerous small segments, which are sequenced using the chain termination method to obtain reads. Multiple overlapping reads for the target DNA are obtained by performing several rounds of this fragmentation and sequencing. Computer programs then use the overlapping ends of different reads to assemble them into a contiguous sequence (Table 1.3).

For example, consider the following two rounds of shotgun reads:

Table 1.3 A simplified example of two rounds of shotgun sequencing (Anderson 1981)

Shotgun stages	Sequenced nucleotides	Relation to original strand
Original strand		**AGCATGCTGCAGTCATGCTTAGGCTA**
First round of shotgun reads	**AGCATGCTGCAG** **TCATGCTTAGGCTA**	**AGCATGCTGCAG**TCATGCTTAGGCTA AGCATGCTGCAG**TCATGCTTAGGCTA**
Second round of shotgun reads	**TTAGGCTA** **AGCATGCTGCAGTCATGC**	AGCATGCTGCAGTCATGC**TTAGGCTA** **AGCATGCTGCAGTCATGC**TTAGGCTA

In this extremely simplified example, the four reads can be assembled into the original sequence using the overlap of their ends to align and order them. However, assembly of complex genomes is additionally complicated by the abundance of

repetitive sequence, meaning similar short reads could come from completely different parts of the sequence. Many overlapping reads for each segment of the original DNA are necessary to overcome these difficulties and accurately assemble the sequence.

1.7.5 Whole-Genome Shotgun Sequencing

Whole-genome shotgun sequencing (Edwards et al. 1990; Edwards and Caskey 1991; Fleischmann 1995) known also as double-barrel shotgun sequencing is an application of pair-wise end sequencing (Roach et al. 1995), from both the ends of a fragment of DNA. Although sequencing both ends of the same fragment and keeping track of the paired data were more cumbersome than sequencing a single end of two distinct fragments, the knowledge that the two sequences were oriented in opposite directions and were about the length of a fragment apart from each other was valuable in reconstructing the sequence of the original target fragment. To apply the strategy, high-molecular-weight DNA is sheared into random fragments, size-selected (usually 2, 10, 50, and 150 kb), and cloned into an appropriate vector. The clones are then sequenced from both ends using the chain termination method yielding two short sequences. Each sequence is called an end-read or read, and two reads from the same clone are referred to as mate pairs. Since the chain termination method usually can only produce reads between 500 and 1000 bases long, in all but the smallest clones, mate pairs will rarely overlap.

The original sequence is reconstructed from the reads using sequence assembly software. First, overlapping reads are collected into longer composite sequences known as contigs. Contigs can be linked together into scaffolds by following connections between mate pairs. The distance between contigs can be inferred from the mate pair positions if the library size is known and has a narrow window of deviation.

Coverage is the average number of reads representing a given nucleotide in the reconstructed sequence. It can be calculated from the length of the original genome (G), the number of reads (N), and the average read length (L) as NL/G. For example, a hypothetical genome with 2000 base pairs reconstructed from 8 reads with an average length of 500 nucleotides will have 2× coverage.

With this approach, it is possible to sequence the whole genome at once using large arrays of sequencers, which makes the whole process much more efficient than more traditional approaches.

1.8 Gene Libraries

Gene libraries store genetic information. These libraries are composed of fragments of donor DNA which are protected by insertion into cloning vectors. Recombinant cloning vectors from a library, inserted into host cells, allow replication of the donor

genetic material. Donor genetic information comes primarily from two sources: (1) Genomic DNA: It is the entire complement of DNA in a donor cell. Eukaryotic genes composed of genomic DNA consist of noncoding introns interspersed among coding exons. (2) cDNA (complementary DNA): Genes composed of cDNA are created using reverse transcriptase to synthesize a DNA copy of an mRNA template. Since mRNA template has already undergone posttranscriptional modifications, it consists solely of coding exons. Therefore, cDNA differs from genomic DNA in that it is composed of exons alone.

1.9 Preparation of cDNA

cDNA reduces the effective size of the eukaryotic cells since it represents only the exon portion of the gene. The lack of introns in eukaryotic cDNA allows its successful translation by bacterial cells, as these cells are unable to remove noncoding introns from the mRNA which is transcribed from a genomic DNA fragment.

The use of cDNA makes it easier to identify a particular target. By isolating mRNA from appropriate cells (e.g., mRNA complementary to fibroin gene from silk gland cells), the probability of locating a target molecule increases.

mRNA is isolated from a host cell by passing a preparation of nucleic acid over a cellulose column, which has been treated with short lengths of thymine deoxyribonucleotides. This type of column is called oligo-dT cellulose column. The poly(A) tail which characterizes the mRNA molecules binds to the complementary thymine nucleotides attached to the cellulose of the column. In this way the mRNA molecules remain within the column, while others containing nucleic acids pass out through the column. The bound mRNA molecules can be chemically removed from the cellulose. cDNA is synthesized from mRNA templates using the retroviral enzyme called reverse transcriptase.

1.10 DNA Fingerprinting (or Genetic Fingerprinting or DNA Testing or DNA Typing or DNA Profiling)

DNA fragments produced by restriction endonucleases can be separated on the basis of size by the technique of gel electrophoresis. Agarose gel electrophoresis (AGE) separates DNA fragments which differ from one another by at least 30–50 nucleotide pairs in length. Polyacrylamide gel electrophoresis (PAGE) separates fragments which differ even by a single nucleotide.

Not the entire length of DNA contains useful information. A large amount of DNA is not translated into useful proteins. It is called “noncoding” or “junk” DNA. Changes often crop up within these regions of junk DNA because they make no contribution to the health or survival of the organism; therefore, it remains in the genome and may inherit to the next generation. If a change occurs within a “coding sequence of DNA” (essential gene), preventing it from working properly, the

organism will not probably survive, effectively removing that altered gene from the population. For this reason, random variations crop up in the noncoding (junk) DNA sequences as often as once in every 200 DNA letters.

The base sequences of the chromosomes of different individuals of the same species closely resemble one another. However, some differences, called polymorphisms, do exist. Each individual has enough polymorphic sites to make its DNA unique. The analysis of various polymorphisms in any one organism will provide a unique profile which can be used to identify it. This is called DNA fingerprinting which was described by Sir Alec Jeffreys in 1985 (Jeffreys et al. 1991).

1.11 Protection of DNA Fragments

Donor genetic sequences, either genomic or cDNA, must be protected from degradation and transported into appropriate host cells by cloning vectors. Cloning vectors are lengths of DNA which generally have three properties: (1) unique recognition sequences, (2) selectable visible markers, and (3) can replicate. The three types of cloning vectors are (1) bacterial plasmids, (2) bacteriophage chromosomes, and (3) cosmids:

1. *Plasmid vectors* often contain genes for antibiotic resistance and genes that govern their transmissions from cell to cell during the process of conjugation. Most plasmids contain a polylinker or multiple cloning site (MCS), which is a short region containing several commonly used restriction sites allowing the easy insertion of DNA fragments at this location. The replication of plasmids can either be linked to the replication of host chromosome "stringent plasmid" or independent of host chromosome "relaxed plasmids." The number of relaxed plasmids per host cell increased by the technique known as amplification.
2. *Bacteriophage (or phage) vector* is a virus that can infect bacteria. Bacteriophage vectors have linear double-stranded DNA molecules, which are flanked by complementary single-stranded sequences, of bases known as "cos sites." The cos sites can bind to one another thus making the phage chromosome circular. Phage particles inject their DNA into host bacterial cells. The phage DNA immediately directs the host bacterial cells to synthesize new phage particles in the lytic process or becomes relatively inert through incorporation into the host chromosome, in the lysogenic process.
3. *Shuttle vectors* have both yeast and bacterial origins of replication and can therefore be maintained in both cell types. This property has allowed the identification of genes within the yeast cell itself. In this system, a yeast DNA library is made and propagated in *E. coli* cells. When sufficient plasmid DNA is available, those plasmids are then isolated from the *E. coli* cells and introduced into yeast cells with known mutations. Donor DNA fragments of about 4000–20,000 base pairs can be incorporated into plasmid or phage vector, respectively.
4. *Cosmid vectors* represent *hybrid vectors* consisting of the phage cos site incorporated into a plasmid molecule. Cosmid vectors can accept donor DNA frag-

ments of 35,000–45,000 base pairs in length. The same endonuclease then linearizes the cosmid in such a way as to leave the cos site intact. Both the cosmid and donor fragments are mixed and allowed to ligate. Some ligation products consist of a donor fragment flanked by two cosmids and therefore have two cos sites. Sequence located between two cos sites can be packaged into a phage particle ready to infect a host cell. The donor DNA can thus be inserted into host cell via the process of infection.

5. *An episome* is a plasmid that can integrate itself into the chromosomal DNA of the host organism (Fig. 1.2). Therefore, it can stay intact for a long time, be duplicated with every cell division of the host, and become a basic part of its genetic makeup. This term is no longer commonly used for plasmids, since it is now clear that a region of homology with the chromosome such as a transposon makes a plasmid into an episome. In mammalian systems, the term episome refers to a circular DNA (such as a viral genome) that is maintained by noncovalent tethering to the host cell chromosome.
6. *F-plasmid*, also known as the fertility F-plasmid or the F-factor, is found in bacteria allowing bacterial conjugation (where genetic information is exchanged) between different bacterial cells. A bacterial cell is described as F+ (male) when it contains this plasmid and F− (female) when it does not. The F-plasmid is also an episome and can integrate into the cell's circular genome; in this instance, a cell would be described as Hfr. When an F+ cell conjugates with an F− cell, the result is two F+ cells, both capable of transmitting the plasmid further by conjugation. In the case of Hfr, the result is one Hfr and one F− cell. The F-plasmid has also been engineered so it contains inserted foreign DNA, called a fosmid.
7. *Fosmids* are similar to cosmids but are based on the bacterial F-plasmid. The cloning vector is limited, as a host (usually *E. coli*) can only contain one fosmid molecule. Low copy number offers higher stability than comparable high copy number cosmids. Fosmid clones were used to help assess the accuracy of the Public Human Genome Sequence.
8. *R-plasmid*, resistance plasmid, is a conjugative factor in bacterial cells that promotes resistance to agents such as antibiotics, metal ions, ultraviolet radiation, and bacteriophages.
9. *Bacterial artificial chromosome (BAC)* is a DNA construct, based on a fertility plasmid (or F-plasmid), used for transforming and cloning in bacteria, usually *E. coli.* F-plasmids play a crucial role because they contain partition genes that promote the even distribution of plasmids after bacterial cell division. The bacterial artificial chromosome's usual insert size is 150 kbp, with a range from 100 to 300 kbp. A similar cloning vector, called a PAC, has also been produced from the bacterial P1-plasmid.

BACs are often used to sequence the genetic code of organisms in genome projects, for example, the Human Genome Project. A short piece of the organism's DNA is amplified as an insert in BACs and then sequenced. Finally, the sequenced parts are rearranged, resulting in the genomic sequence of the organism.

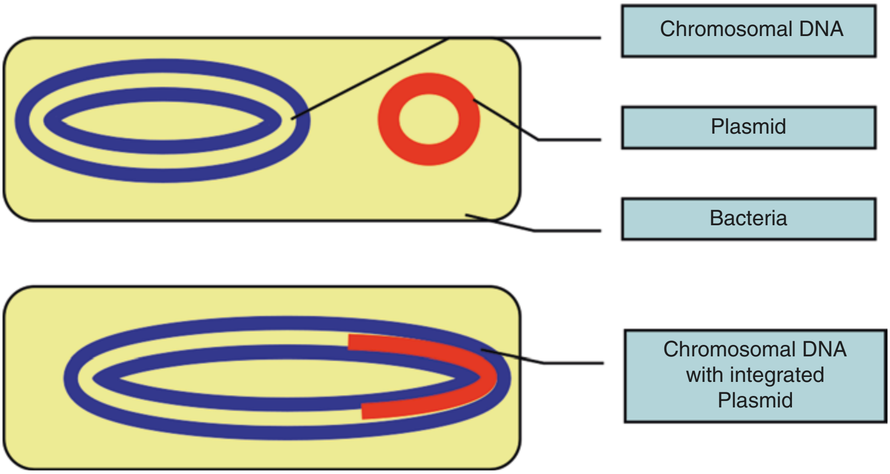


Fig. 1.2 (*Above*) Bacterial cell with chromosomal DNA of bacteria and plasmid DNA. (*Below*) Plasmid DNA integrated with the chromosomal DNA

BACs can carry both the gene and various promoter sequences which can often show the genes' true expression level. They are transferred over to the organisms by electroporation/transformation or transfection with a suitable virus or microinjection. BACs can also be utilized to detect genes or large sequences of interest and then used to map them onto the human chromosome using BAC arrays.

1.12 Contig

In shotgun DNA sequencing projects, a contig (from contiguous) (Staden 1979) is a set of overlapping DNA segments derived from a single genetic source. A contig in this sense can be used to deduce the original DNA sequence of the source. A contig map depicts the relative order of a linked library of contigs representing a complete chromosome segment.

1.13 Expressed Sequence Tag (EST)

An expressed sequence tag (EST) is a subsequence of a transcribed spliced nucleotide sequence (either protein coding or not). They are intended as a way to identify gene transcripts and are instrumental in gene discovery and gene sequence determination. The identification of ESTs has proceeded rapidly, with approximately 42 million ESTs now available in public databases (e.g., GenBank 3/2007, all species).

An EST is produced by one-shot sequencing (single pass) of a cloned mRNA (i.e., sequencing several hundred base pairs from an end of a cDNA clone taken

from a cDNA library). The resulting sequence is a relatively low-quality fragment whose length is limited by current technology to approximately 500–800 nucleotides. Because these clones consist of DNA that is complementary to mRNA, the ESTs represent portions of expressed genes. They may be present in the database as either cDNA/mRNA sequence or as the reverse complement of the mRNA, the template strand.

ESTs can be mapped to specific chromosome locations using physical mapping techniques, such as radiation hybrid mapping or **FISH** (fluorescent in situ hybridization). Alternatively, if the genome of the organism that originated the EST has been sequenced, one can align the EST sequence to that genome.

ESTs are the tools to refine the predicted transcripts for those genes, which leads to prediction of their protein products and eventually of their function. Moreover, the situation in which those ESTs are obtained (tissue, organ, disease state—e.g., cancer) gives information on the conditions in which the corresponding gene is acting. ESTs contain enough information to permit the design of precise probes for DNA microarrays that then can be used to determine the gene expression.

To generate ESTs, first a cDNA library is created from cell or tissue, and then hundreds or thousands of clones are picked from the library and sequenced in just one pass, without validation, without full-length sequence, usually from poly(A) tail, but can also be sequenced from 5′ or middle.

1.14 Transposons

Transposons are sequences of DNA that can move around to different positions within the genome of a single cell, a process called transposition. In the process, they can cause mutations and change the amount of DNA in the genome. Transposons are also called “jumping genes” or “mobile genetic elements.” There are a variety of mobile genetic elements, and they can be grouped based on their mechanism of transposition. Class I mobile genetic elements, or retrotransposons, move in the genome by being transcribed to RNA and then back to DNA by reverse transcriptase, while class II mobile genetic elements move directly from one position to another within the genome using a transposase to “cut and paste” them within the genome. Transposons are very useful to researchers as a means to alter DNA inside of a living organism. Transposons make up a large fraction of genome sizes which is evident through the C-values of eukaryotic species. For example, about 45% of the human genome is composed of transposons and their defunct remnants.

1.15 Cloning

Donor genetic sequences of cDNA are cloned to get recombinant vectors. Once a recombinant vector has been synthesized, it must be inserted into the target host cell (e.g., bacterial cell) in order to allow replication of the exogenous genetic material (Fig. 1.3) Watson JD (2007).

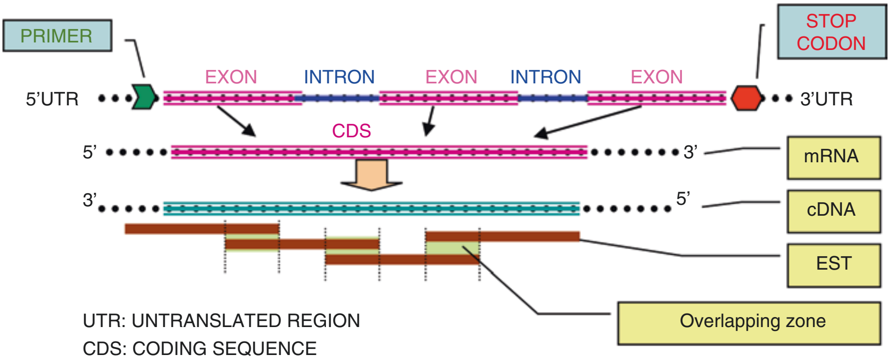


Fig. 1.3 DNA to mRNA, mRNA to cDNA. ESTs are parts of cDNA

Treatment of linearized cloning vector with alkaline phosphatase removes the 5′ phosphate group, necessary for the religation of the cleaved ends. Thus, pretreatment of the cleaved vector with this enzyme ensures that only recombinant vectors result when a preparation of cleaved vector molecules is incubated with a preparation of donor DNA fragments in the presence of DNA ligase because the end of treated vector will be unable to ligate to one another.

Cells which are able to take up exogenous DNA, including recombinant plasmids, are called competent cells. Some cell types are naturally competent, while other cell types are not. *E. coli* cells must be treated with calcium chloride and heat shock or a similar procedure to transform them to a competent state. Cells which have taken up recombinant plasmid DNA are called transformed cells. The efficiency of transformation is generally extremely low and ranges from 0.1 to 10%.

E. coli cells are often chosen as host cells for a number of reasons. The *E. coli* chromosome has been well characterized, and many of its polypeptide products are identified. *E. coli* cells are easy to grow in laboratory and have a fast multiplication rate of 20–60 min.

Neither type of gene libraries (genomic DNA nor cDNA) has an index and must therefore be analyzed (screen) with specific probe molecules to find and identify target sequences.

1.15.1 Identification of Transformed Cell

Transformed cells can often be identified with the use of a selective media which interacts in some way with a selectable marker located on the cloning vector. Typically the cloning vector carries gene which confers drug resistance on a transformed cell, thus allowing only transformed cells to grow in selective media containing that particular drug. Non-transformed cells cannot grow in the selective medium.

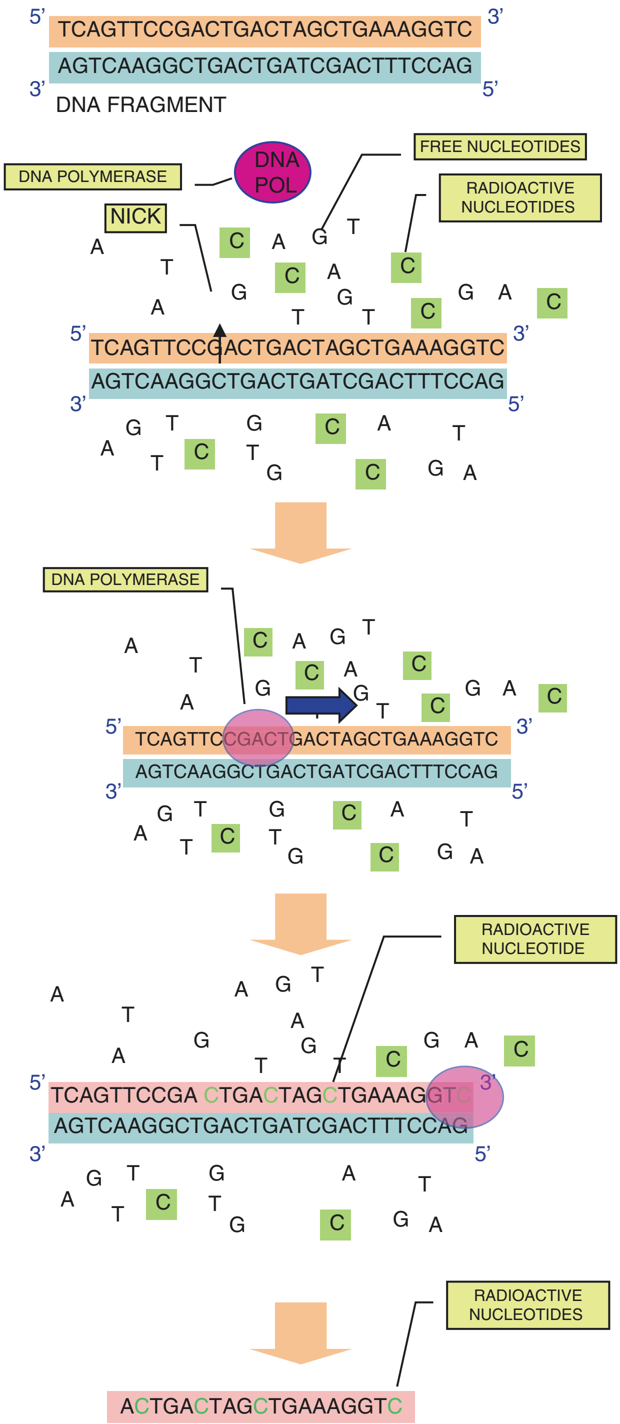


Fig. 1.4 Stages in synthesizing a *Probe*

The presence of an inserted donor gene in the cloning vector can sometimes be determined by insertional inactivation. This is the loss of a gene product due to insertion of foreign DNA in a recognition sequence located within a particular gene.

The basic screening techniques include the transfer of the host cells or the phage vector to a solid substrate, usually nitrocellulose membrane (or nylon or PVDF) through blotting, subsequent treatment of the sheet with the labeled probe which is complementary to the target sequence. Probes can be cDNA, genomic DNA, RNA, or antibody molecules. Specific probes are chosen based on the information known about the target sequence. Probes will hybridize to complementary molecules on the substrate. Hybridized probe/target complexes are visualized with autoradiography. Alternatively, mRNA probes can be removed from the target molecule and used to direct protein synthesis in order to identify the original gene sequence.

1.16 DNA Probe

1. DNA to be made radioactive (radiolabeled) is put into a tube.
2. *Nicks*, or horizontal breaks, are introduced along a strand into the DNA to be radiolabeled. At the same time, individual nucleotides are added to the nicked DNA, one of which, **C**, is radioactive (Fig. 1.4).
3. *DNA polymerase* is added to the tube with the nicked DNA and the individual nucleotides. The DNA polymerase will become immediately attracted to the nicks in the DNA and attempt to repair the DNA, starting from the 5′ end and moving toward the 3′ end.
4. The DNA polymerase begins repairing the nicked DNA. It destroys all the existing bonds in front of it and places the new nucleotides, gathered from the individual nucleotides mixed in the tube, behind it. Whenever a G base is read in the lower strand, a radioactive **C** base is placed in the new strand. In this fashion, the nicked strand, as it is repaired by the DNA polymerase, is made radioactive by the inclusion of radioactive **C** bases.
5. The nicked DNA is then heated, splitting the two strands of DNA apart. This creates single-stranded radioactive and nonradioactive pieces. The *radioactive DNA*, *now called a probe*, is ready for use.

1.17 Blotting

Southern blotting is the analysis of DNA sequences with either a DNA or RNA probe. Northern blotting is the analysis of RNA sequences with DNA or RNA probe. Western blotting is the analysis of proteins with an antibody probe. Transformed host cells can be maintained for a long period of time by storage at very cold temperatures, making gene libraries reusable. Frozen cells thawed many years after their original storage behave as if they are freshly prepared. Thus, gene libraries can be created, conserved, probed, regenerated, and re-probed many times.

1.17.1 The Southern Blot

The Southern blot is a method of enhancing the result of an agarose gel electrophoresis by marking specific DNA sequences. The method is named after its inventor, the British biologist Southern, Edwin M (Southern 1975). This became a convention to other blot methods to be named similarly to indicate variants in blot technology, e.g., Northern blot, Western blot, and Southwestern blot (Peters 1993).

DNA strands are broken into fragments by restriction endonucleases. The fragments are then electrophoresed on a gel to separate cut DNA based on size. If DNA is larger than 15 kb, prior to blotting, the gel may be treated with a dilute acid, such as dilute HCl, which acts to depurinate the DNA fragments. This breaks the DNA into smaller pieces that will be able to complete the transfer more efficiently than larger fragments.

DNA bands are transferred to nylon sheet: The gel from the DNA electrophoresis is treated with an alkaline solution (typically containing sodium hydroxide) to cause the double-stranded DNA to denature, separating it into single strands. Denaturation is necessary so that the DNA will stick to the membrane and be hybridized by the probe. Since the gel is brittle, it cannot withstand further process. Therefore, the bands are transferred to a firm supporting sheet (substrate or membrane) (Towbin et al. 1979). A sheet of nitrocellulose (or nylon, polyvinylidene fluoride (PVDF, it is also known as KYNAR®)) membrane is placed on top of the gel. Pressure is applied evenly to the gel (either using suction or by placing a stack of paper towels and a weight on top of the membrane and gel). This causes the DNA to move from the gel onto the membrane by capillary action, where it sticks. The membrane is then baked (in the case of nitrocellulose) or exposed to ultraviolet radiation (nylon) to permanently cross-link the DNA to the membrane.

The sheet is then treated with a hybridization probe—an isolated DNA molecule with a specific sequence that pairs with the appropriate sequence (the appropriate sequence is the complementary sequence of what the restriction enzyme recognized). The probe DNA is labeled so that it can be detected, usually by incorporating radioactivity or tagging the molecule with a fluorescent or chromogenic dye. In some cases, the hybridization probe may be made from RNA, rather than DNA.

After hybridization, excess probe is washed from the membrane, and the pattern of hybridization is visualized on X-ray film by autoradiography in the case of a radioactive or fluorescent probe or by development of color on the membrane itself if a chromogenic detection is used. When making use of hybridization in the laboratory, DNA must first be denatured, usually by using heat or chemicals. Denaturing is a process by which the hydrogen bonds of the original double-stranded DNA are broken, leaving a single strand of DNA whose bases are available for hydrogen bonding.

Once the DNA has been denatured, a single-stranded radioactive probe can be used to see if the denatured DNA contains a sequence similar to that on the probe. The denatured DNA is put into a plastic bag along with the probe and some saline liquid; the bag is then shaken to allow sloshing. If the probe finds a fit, it will bind to the DNA (Fig. 1.5).

The fit of the probe to the DNA does not have to be exact. Sequences of varying homology (Fig. 1.6) can stick to the DNA even if the fit is poor; the poorer the fit, the fewer the hydrogen bonds between the probe and the denatured DNA. The ability of low-homology probes to still bind to DNA can be manipulated through varying the temperature of the hybridization reaction environment or by varying the amount of salt in the sloshing mixture.

1.17.2 The Northern Blot

The Northern blot (Alwine et al. 1977) is a technique to study gene expression. It is similar to the Southern blot procedure, with the fundamental difference that RNA, rather than DNA, is the substance being analyzed by electrophoresis and detection with a hybridization probe. A notable difference in the procedure (as compared with the Southern blot) is the addition of formaldehyde in the agarose gel, which acts as a denaturant. As in the Southern blot, the hybridization probe may be made from DNA or RNA. A variant of the procedure known as the reverse Northern blot was occasionally used. In this procedure, the substrate nucleic acid (i.e., affixed to the membrane) is a collection of isolated DNA fragments, and the probe is RNA extracted from a tissue and radioactively labeled.

The use of DNA microarrays that have come into widespread use in the early 2000s is similar to the reverse procedure, in that they involve the use of isolated DNA fragments affixed to a substrate and hybridization with a probe made from cellular RNA. Thus, the reverse procedure enabled the one-at-a-time study of gene expression using Northern analysis to evolve into gene expression profiling, in which many of the genes in an organism may have their expression monitored.

1.17.3 The Western Blot (Immunoblot)

A Western blot (immunoblot) (Burnette 1981) is a method to detect protein in a given sample of tissue homogenate or extract. It uses gel electrophoresis to separate denatured proteins by mass. The proteins are then transferred out of the gel and onto a membrane (nitrocellulose or PVDF), where they are "probed" using antibodies specific to the protein (Renart et al. 1979). As a result, researchers can examine the size, processing, or amount of protein in a given sample and compare several groups.

Other related techniques include using antibodies to detect proteins in tissues (immunohistochemistry) and cells (immunocytochemistry) or the use of antibody to separate proteins by precipitation (immunoprecipitation).

1.17.3.1 Tissue Preparation

Typically, samples are taken from either tissue or from cell culture. The samples are cooled or frozen rapidly. They are homogenized using sonication or mechanical force or simply lysed using high-salt buffers (150 mM). The resulting "whole-cell

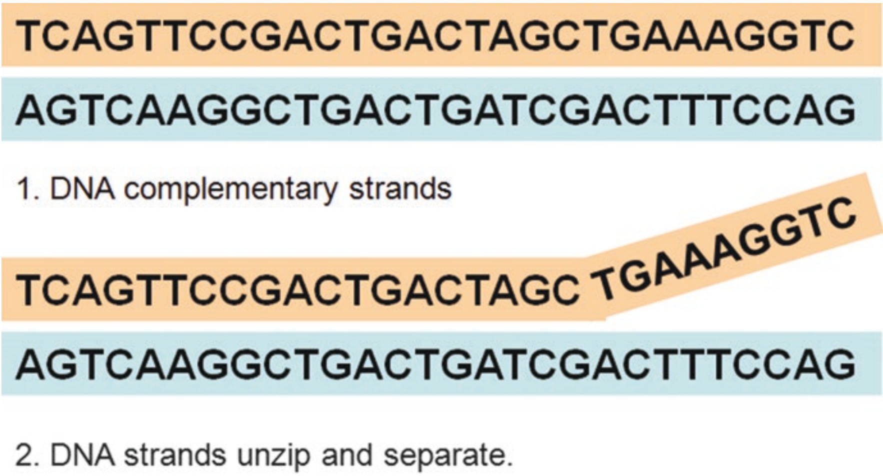


Fig. 1.5 Hybridization of radioactive probe with the single-stranded DNA

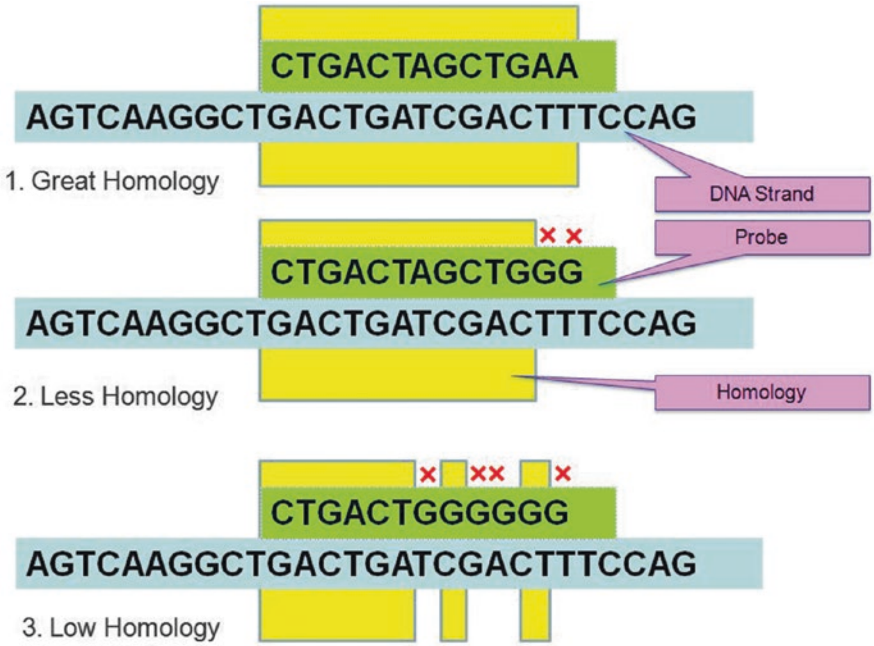


Fig. 1.6 Varying degree of homology between the DNA strand and the probe

homogenate" or "whole-cell fraction" can be used as is or subjected to centrifugation in a series of steps to isolate cytosolic (cell interior), nuclear, and membrane fractions. The prepared sample is then assayed for protein concentration so that a consistent amount of protein can be taken from each different sample.

1.17.3.2 Protein Sample Preparation

Samples are boiled from 1 to 5 min in a buffer solution (e.g., Laemmli's buffer—known as "sample buffer"), containing a buffer substance, normally *tris* base, a dye, a sulfhydryl compound (typically beta-mercaptoethanol or dithiothreitol (DTT) (for reducing disulfide bonds)), an anionic lipophilic detergent (sodium dodecyl sulfate—SDS), and a glycerol to increase its buoyant density. The boiling denatures the proteins, unfolding them completely. The SDS then surrounds the protein with a negative charge, and the beta-mercaptoethanol prevents the reformation of disulfide bonds. The glycerol increases the density of the sample versus the upper buffer in the gel tank and thus facilitates loading the samples as they will sink to the bottom of the gel pockets.

1.17.3.3 Separation of Protein Fractions

The Proteins of the sample are separated according to molecular weight using polyacrylamide gel electrophoresis, it is also possible to use a 2-D gel which spreads the proteins from a single sample out in two dimensions, and proteins are separated according to isoelectric point (pI at which they have neutral net charge) in the first dimension and according to their molecular weight in the second dimension.

In order to make the proteins accessible to antibody detection, they are moved from within the gel onto a membrane of nitrocellulose or PVDF. The membrane is placed face to face with the gel, and current is applied to large plates on either side. The charged proteins move from within the gel onto the membrane while maintaining the organization they had within the gel. As a result of this "blotting" process, the proteins are exposed on a thin surface layer for detection. Both varieties of the membrane are chosen for their nonspecific protein-binding properties (i.e., binds all proteins equally well). Protein binding is based upon hydrophobic interactions, as well as charged interactions between the membrane and protein. Nitrocellulose membranes are economical than PVDF but are far more fragile and do not stand up well to repeated probing.

Since the membrane has been chosen for its ability to bind protein and both antibodies and the target are proteins, steps must be taken to prevent interactions between the membrane and the antibody used for detection of the target protein. Blocking of nonspecific binding is achieved by placing the membrane in a dilute solution of protein—typically bovine serum albumin (BSA) or nonfat dry milk—with a minute percentage of detergent such as Tween 20. The protein in the dilute solution attaches to the membrane in all places where the target proteins have not attached. Thus, when the antibody is added, there is no room on the membrane for it to attach other than on the binding sites of the specific target protein. This reduces

"noise" in the final product of the Western blot, leading to clearer results, and eliminates false positives.

During the detection process, the membrane is "probed" for the protein of interest with antibodies and links them to a reporter enzyme, which drives a colorimetric or photometric signal. This process takes place in a two-step method (now one-step method is also available for certain applications).

1.17.3.4 Two-Step Method

1. First Step: Primary Antibody—Antibodies are generated when a host species or immune cell culture is exposed to the protein of interest (or a part thereof). Normally a part of the immune response, here they are harvested and used as sensitive and specific detection tools that bind the protein directly—hence called primary antibody.

After blocking, a dilute solution of primary antibody (~0.5–5 μg/mL) is incubated with the membrane with gentle agitation. Typically, the solution is comprised of buffered saline solution with a small percentage of detergent and sometimes with powdered milk or BSA. The antibody solution and the membrane can be sealed and incubated together for 30 min to overnight. It can also be incubated at different temperatures, with warmer temperatures being associated with more binding, both specific to the target protein (called "signal") and nonspecific (called "noise").

2. Second Step: Secondary Antibody—After rinsing the membrane to remove unbound primary antibody, it is exposed to another antibody, directed at a species-specific portion of the primary antibody. This is known as a secondary antibody and, due to its targeting properties, generally referred to as "anti-mouse," "anti-goat," etc. Antibodies come from animal sources (or animal-sourced hybridoma cultures); an anti-mouse secondary antibody will bind to just about any mouse-sourced primary antibody. The secondary antibody is usually linked to biotin or to a reporter enzyme such as alkaline phosphatase or horseradish peroxidase. This step confers an advantage in that several secondary antibodies will bind to one primary antibody, providing enhanced signal.

1.17.3.5 One-Step Method

This requires a probe antibody which both recognizes the protein of interest and contains a detectable label, probes which are often available for known protein tags. The primary probe is incubated with the membrane in a manner similar to that for the primary antibody in a two-step process and then is ready for direct detection after a series of wash steps.

After the unbound probes are washed away, the Western blot is ready for detection of the probes that are labeled and bound to the protein of interest. In practical terms, not all Westerns reveal protein only at one band in a membrane. Size

approximations are taken by comparing the stained bands to that of the marker or ladder loaded during electrophoresis. The process is repeated for a structural protein, such as actin or tubulin, that should not change between samples. The amount of target protein is indexed to the structural protein to control between groups. This practice ensures correction for the amount of total protein on the membrane in case of errors or incomplete transfers.

1.17.3.6 Colorimetric Detection

The colorimetric detection method depends on incubation of the Western blot with a substrate that reacts with the reporter enzyme (such as peroxidase) that is bound to the secondary antibody. This converts the soluble dye into an insoluble form of a different color that precipitates next to the enzyme and thereby stains the nitrocellulose membrane. Development of the blot is then stopped by washing away the soluble dye. Protein levels are evaluated through densitometry or spectrophotometry. ELISPOT (the enzyme-linked immunosorbent spot) and ELISA (the enzyme-linked immunosorbent assay) are popular examples.

1.17.3.7 Chemiluminescence

Chemiluminescent detection methods depend on incubation of the Western blot with a substrate that will luminesce when exposed to the reporter on the secondary antibody. The light is then detected by photographic film and more recently by CCD cameras which capture a digital image of the Western blot. The image is analyzed by densitometry, which evaluates the relative amount of protein staining and quantifies the results in terms of optical density. Newer software allows further data analysis such as molecular weight analysis if appropriate standards are used. The "enhanced chemiluminescent" (ECL) detection is considered to be among the most sensitive detection methods for blotting analysis.

1.17.3.8 Radioactive Detection

Radioactive labels do not require enzyme substrates, but rather allow the placement of medical X-ray film directly against the Western blot which develops as it is exposed to the label and creates dark regions which correspond to the protein bands of interest (see image to the right). The importance of radioactive detection methods is declining, because it is very expensive, health and safety risks are high, and ECL provides a useful alternative.

1.17.3.9 Fluorescent Detection

The fluorescently labeled probe is excited by light, and the emission of the excitation is then detected by a photo sensor such as CCD camera equipped with appropriate emission filters which captures a digital image of the Western blot and allows further data analysis such as molecular weight analysis and a quantitative Western blot analysis. Fluorescence is considered to be among the most sensitive detection methods for blotting analysis.

1.18 Polymorphism in Repeated Sequences

On chromosomes, there are sequences of repeated DNA nucleotides. The number of repeats can vary from about one to thirty and are not the same from individual to individual. These sequences are called variable number tandem repeats (VNTRs). Within the VNTRs, there are sites where an enzyme can cut the DNA, and the location of these sites also varies from person to person. Cutting with the enzyme will lead to DNA fragments of different lengths, which are called Restriction Fragment Length Polymorphisms (RFLPs) (Williums et al. 1990).

1.19 DNA Markers

Markers are DNA sequences that can be identified by a simple assay, allowing the presence or absence of neighboring stretches of genome to be inferred. Markers may be short, such as single base pair change (single nucleotide polymorphism), or long such as DNA fragment generated by restriction digestion. DNA markers can be identified directly, e.g., by DNA sequencing or indirectly as in case of allozymes.

1.19.1 Types of Markers

1. Non-PCR-based markers
   a. RFLP
2. PCR-based markers
   a. RAPD
   b. AFLP
   c. Minisatellites
   d. Microsatellites (SSR, STR, SSLPs)
   e. SNP
   f. Sequence-tagged sites (SCARs, CAPS, ISSRs)
   g. Diversity arrays
   h. LCN-DNA

1.19.1.1 RFLP

DNA polymorphism can be identified by the ability of various restriction enzymes to cleave DNA in the vicinity of the polymorphism into variable sizes of DNA fragments. This is called Restriction Fragment Length Polymorphisms (RFLPs). When DNA fingerprinting first began, RFLP (Zabeau and Vos 1993) analysis was used. Now it has been almost completely replaced with newer PCR-based techniques. RFLP analysis is performed by using a restriction enzyme to cut the DNA into fragments which are separated into bands by agarose gel electrophoresis. These bands of DNA are transferred by Southern blotting from the agarose gel to a nylon membrane. This is treated with a radioactively labeled DNA probe which binds to certain specific DNA sequences on the membrane. The excess DNA probe is then washed

off. An X-ray film placed next to the nylon membrane detects the radioactive pattern. This film is then developed to make a visible pattern of bands called a DNA fingerprint. By using multiple probes targeting various polymorphisms in successive X-ray images, a fairly high degree of discrimination was possible.

Advantages of RFLPs are as follows: RFLPs have higher level of polymorphism than isozymes, larger number of loci can be identified, produce semidominat markers, allowing determination of homozygosity or heterozygosity, and have selective neutrality. They are stable and reproducible.

Disadvantages of RFLPs are the exact sizes of the bands are unknown and comparison to a molecular weight ladder is done in a purely qualitative manner. RFLP is a very time-consuming method which requires relatively high quantity of good-quality DNA. One has to work with radioisotopes. Too many polymorphisms for a short probe.

1.19.1.2 PCR Based Markers

With the invention of the polymerase chain reaction (PCR) (Mullis 1998; Wolfe and Liston 1998; Wolfe et al. 1998), DNA fingerprinting took huge strides forward in both discriminating power and ability to recover information from very small starting samples. PCR involves the amplification of specific regions of DNA using a cycling of temperature and a thermostable polymerase enzyme along with sequence-specific primers of DNA. Systems such as the HLA-DQ alpha reverse dot blot strips grew to be very popular due to their ease of use and the speed with which a result could be obtained; however, they were not as discriminating as RFLPs. A large number of protocols that are rapid and require only a small quantity of DNA have been developed.

RAPD

RAPD (Random Amplification of Polymorphic DNA) is a type of PCR reaction, but the segments of DNA that are amplified are random. The scientist performing RAPD creates several arbitrary, short primers (8–12 nucleotides) and then proceeds with the PCR using a large template of genomic DNA, hoping that fragments will amplify. By resolving the resulting patterns, a semi-unique profile can be generated from a RAPD reaction.

No knowledge of the DNA sequence for the targeted gene is required, as the primers will bind somewhere in the sequence, but it is not certain exactly where. This makes the method popular for comparing the DNA of biological systems that have not had the attention of the scientific community or in a system in which relatively few DNA sequences are compared (it is not suitable for forming a DNA data bank). Due to the fact that it relies on a large, intact DNA template sequence, it has some limitations in the use of degraded DNA samples. Its resolving power is much lower than targeted, species-specific DNA comparison methods, such as Short Tandem Repeats.

Advantages of RAPDs are they are more polymorphic than RFLPs, simple, and quick and have selective neutrality. *Disadvantages* are they are dominant and do not permit the scoring of heterozygous individuals. Reproducibility is limited.

AFLP

AFLP-PCR, amplified fragment length polymorphism-polymerase chain reaction (Vos et al. 1995), is a highly sensitive method for detecting polymorphisms in DNA. The procedure of this technique is divided into three steps: (1) digestion of total cellular DNA with one or more restriction enzymes and ligation of restriction half-site-specific adaptors to all restriction fragments, (2) selective amplification of some of these fragments with two PCR primers that have corresponding adaptor and restriction site-specific sequences, and (3) electrophoretic separation of amplicons on a gel matrix, followed by visualization of the band pattern.

AFLP is relied on variable number tandem repeat (VNTR) polymorphisms to distinguish various alleles, which were separated on a polyacrylamide gel using an allelic ladder (as opposed to a molecular weight ladder). Bands could be visualized by silver staining the gel. As with all PCR-based methods, highly degraded DNA or very small amounts of DNA may cause allelic dropout (causing a mistake in thinking a heterozygote is a homozygote) or other stochastic effects. In addition, because the analysis is done on a gel, very high number repeats may bunch together at the top of the gel, making it difficult to resolve. AFLP analysis can be highly automated and allows for easy creation of phylogenetic trees based on comparing individual samples of DNA. A variation of AFLP is TE Display, used to detect transposable element mobility.

Advantages: No sequence information required, reliable, highly sensitive and very large number of polymorphisms per reaction, highly reproducible (repeatable), selective neutrality. *Disadvantages*: Null allele not detected, proprietary technology.

Minisatellite

Minisatellite is a section of DNA that consists of a short series of bases 10–100 bp; these occur at more than 1000 locations in the genome. This series usually contains the same central sequence of letters "GGGCAGGAXG" (where X can be any one of A, T, G, C letters). This sequence encourages chromosomes to swap DNA. When this happens, frequent mistakes are made; this causes minisatellites at over 1000 locations in the genome to have slightly different numbers of repeats, thereby making them unique. Due to their high level of polymorphism, minisatellites were extensively used for DNA fingerprinting as well as for genetic markers. Minisatellites have also been implicated as regulators of gene expression (e.g., at levels of transcription, alternative splicing, or imprint control) or as part of bona fide open reading frames. Minisatellites have also been associated with chromosome fragile sites and are proximal to a number of recurrent translocation breakpoints.

Microsatellites: STR, SSR, or SSLPs

The most prevalent method of DNA fingerprinting used today is based on PCR and uses Short Tandem Repeats (STR) or Microsatellites or Simple Sequence Repeats (SSR) or Simple Sequence Length Polymorphisms (SSLPs) (Gupta et al. 1994; Tautz 1989; Zietkiewicz et al. 1994). The lengths of sequences used most often are mono-, di-, tri-, or tetra-nucleotides, e.g., AAAAAAA would be referred

to as $(A)_7$, GTGTGTGTGTGT as $(GT)_7$, CTGCTGCTGCTG as $(CTG)_4$, and ACTCACTCACTCACTCACTC as $(ACTC)_5$. Microsatellites are inherited in a Mendelian fashion. They are typically neutral and codominant and are used as molecular markers. Because different individuals have different numbers of repeat units, these regions of DNA can be used to discriminate between individuals. These STR loci are targeted with sequence-specific primers and are amplified using PCR. The DNA fragments that result are then separated and detected using capillary electrophoresis (CE) and gel electrophoresis (PAGE). The polymorphisms displayed at each STR region are by themselves very common; typically each polymorphism will be shared by around 5–20% of individuals. When looking at multiple loci, it is the unique combinations of these polymorphisms to an individual that makes this method discriminating as an identification tool. The more STR regions that are tested in an individual, the more discriminating the test becomes.

DNA is denatured at a high temperature, separating the double strand. Annealing of primers and the extension of nucleotide sequences along opposite strands are effected at lower temperatures. This process results in production of enough DNA to be visible on agarose or acrylamide gels; only small amounts of DNA are needed for amplification as thermocycling in this manner creates an exponential increase in the replicated segment. With the advance of PCR technology, primers that flank microsatellite loci are simple and quick to use, but the development of correctly functioning primers is a critical process.

Development of Microsatellite Primers

1. For searching specific microsatellite markers in particular regions of a genome, for example, within a particular exon of a gene, primers can be designed manually. This involves searching the genomic DNA sequence for microsatellite repeats, which can be done visually or by using automated tools such as repeat masker. Once the potentially useful microsatellites are determined (removing non-useful ones such as those with random inserts within the repeat region), the flanking sequences can be used to design oligonucleotide primers which will amplify the specific microsatellite repeat in a PCR reaction.
2. Random microsatellite primers can be developed by cloning random segments of DNA from the focal species. These are inserted into a plasmid or phage vector, which is in turn implanted into *Escherichia coli* bacteria. Colonies are then developed and screened with fluorescently labeled oligonucleotide sequences that will hybridize to a microsatellite repeat, if present on the DNA segment. If positive clones can be obtained from this procedure, the DNA is sequenced, and PCR primers are chosen from sequences flanking such regions to determine a specific locus. This process involves significant trial and error on the part of researchers, as microsatellite repeat sequences must be predicted and primers that are randomly isolated may not display significant polymorphism. Microsatellite loci are widely distributed throughout the genome and can be isolated from semi-degraded DNA of older specimens, as all that is needed is a suitable substrate for amplification through PCR.

Microsatellites have been proved to be versatile molecular markers, particularly for population analysis, but they are not without limitations. *Advantages*: High level of polymorphisms, high locus specificity, easy and fast to run, robust and reproducible. *Disadvantages*: May only be used for intraspecific and intragenomic alleles, time-consuming and expensive.

SNP (Single Nucleotide Polymorphism)

SNP (pronounced as snip) is a DNA sequence variation occurring when a single nucleotide—A, T, C, or G—in the genome (or other shared sequences) differs between members of a species (or between paired chromosomes in an individual). For example, two sequenced DNA fragments from different individuals, AAGCCTA to AAGCTTA, contain a difference in a single nucleotide. In this case, it is referred as two alleles: C and T. Almost all common SNPs have only two alleles.

Within a population, SNPs can be assigned a minor allele frequency—the ratio of chromosomes in the population carrying the less common variant to those with the more common variant. SNPs may fall within coding sequences of gene and noncoding regions of genes or in the intergenic regions between genes. SNPs within a coding sequence will not necessarily change the amino acid sequence of the protein that is produced, due to degeneracy of the genetic code. A SNP in which both forms lead to the same polypeptide sequence is termed synonymous (sometimes called a silent mutation)—if a different polypeptide sequence is produced, they are non-synonymous. SNPs that are not in protein coding regions may still have consequences for gene splicing, transcription factor binding, or the sequence of noncoding RNA.

STS (Sequence Tagged Sites)

From the sequence information, oligonucleotide primers 18–20 nucleotide long are synthesized that are complementary to each end of the RAPD product or the clone. These new primers are then used to amplify DNA by PCR. Two results could occur. First the size of the amplification products among different DNAs (e.g., two parents differing from disease resistance locus) could be polymorphic. Alternatively the amplification product could be monomorphic (of the same size). If this is the case, then it will be necessary to cut the products with various restriction enzymes to identify polymorphisms. SCAR, CAPS, and ISSRs are grouped under this category.

SCAR (Sequence Characterized Amplified Region)

The RAPD band from the +allele (i.e., the allele that gives the band) is cloned and sequenced. Longer primers a1 and a2 are synthesized. Because they contain longer sequences, they are likely to be specific only for the desired locus and will not amplify any other loci. However, it is also likely that a1 and a2 will amplify both the + and −alleles at that locus. If so then it should be possible to amplify the + and − alleles. By comparing the sequences of the + and −allele, it should be possible to find out other mutations, internal to the a1 and a2, primers which are polymorphic between two alleles. If such polymorphism can be identified, then it should be possible to synthesize new primers, a1 and a2, that will only amplify a region of the +allele but not the −allele. 16–24 bp primers designed from the ends of cloned

RAPD markers are used. This technique converts a band which is prone to difficulties in interpretation and/or reproducibility into a very reliable marker.
Advantages: Simpler pattern than RAPDs, robust and reproducible, Mendelian inheritance, sometimes convertible to codominant markers. *Disadvantages*: Require a small degree of sequence knowledge, require effort and expense in designing specific primers for each locus.

CAPS (Cleaved Amplified Polymorphic Sequence)

This method is based on the design of specific primers, amplification of DNA fragments, and generation of smaller possibly variable fragments by means of a restriction enzyme. This technique aims to convert an amplified band that does not show variation into a polymorphic one. In this method, A band, DNA, gene sequence, or another type of markers is identified as important. Either the band is detected through PCR and cut out of the gel and fragment cloned and sequenced, or the fragment sequence is already available. Specific primers are designed from fragment sequences. The newly designed primers are used to amplify the template DNA. The PCR product is subjected to digestion by a panel of restriction enzymes. Polymorphism may be identified with some of the enzymes.
Advantages: Robust assay, because of long primers; codominant markers; can be compared with library markers. *Disadvantages*: Require at least small amount of sequence knowledge, effort, and expense to produce specific primers.

ISSRs (Inter-Simple Sequence Repeats)

ISSR techniques (Wolfe et al. 1998; Bornet and Branchard 2001) are nearly identical to RAPD techniques except that ISSR primer sequences are designed from microsatellite regions and the annealing temperatures used are higher than those used for RAPD markers. They are the regions found between microsatellite repeats. Technique is based on PCR amplification of intermicrosatellite sequences. It targets multiple loci because of the known abundance of repeat sequence spread all over the genome. In this method, a typical PCR is performed in which primers have been designed, based on a microsatellite repeat sequence, and extended one to several bases into the flanking sequence as anchor points. Different alternatives are possible: Only one primer is used; two primers of similar characters are used; combinations of microsatellite-sequence-anchored primer with a random primer (i.e., those used for RAPD).

Advantages: Do not require prior sequence information. Variation within unique regions of genome may be found at several loci simultaneously. Microsatellite sequence specific. Very useful DNA fingerprinting especially for closely related species. *Disadvantage*: Only dominant markers can be identified.

DArT (Diversity Array Technology)

DArT can detect and type DNA variation at several hundred genomic loci in parallel without relying on sequence information. Two steps are involved in this method. (1) Generation of array: Restriction-generated fragments representing the diversity of a genepool are cloned. The outcome is called representation (typically 0.1–10%) of

the genome. Polymorphic clones in the library are identified by arraying insert from a random set of clones and hybridizing the array to different samples. The inserts from polymorphic clones are immobilized on a chip. (2) Genotyping a sample: Label the representation (DNA) of the sample with fluorescence and hybridize against the array. Scan the array and measure for each spot the amount of hybridization signal. By using multiple labels, contrast a representation from one sample with the other or with control probe.

Advantages: Do not require sequence information, high output, fast data acquisition and analysis, detects single base changes as well as insertions and/or deletions, detects differences in DNA methylation, depending on the enzyme used to generate the fragments, small DNA sample is enough, good transferability of markers among breeding populations, full automation possible. *Disadvantages*: Dominance of markers, technically demanding, low polymorphism in genomic library.

LCN-DNA (Low Copy Number DNA)

The journal *Nature* summarizes this technique as “Initial tests showed that they could readily obtain correct genetic profiles from swabs taken directly from the palm of a hand (13 of 13)” (Findlay et al. 1997; Ronald et al. 1997; van Oorschot et al. 2005). DNA yields varied from 2 to 150 ng (average 48.6 ng). Dry hands and those that had been washed recently tended to provide the least DNA. This is similar to ISSR, but a small trace of DNA is sufficient.

Advantages: Work with <100 pg genomic DNA (~15–17 diploid copies of nuclear DNA markers such as STRs) below stochastic threshold level where PCR amplification is not as reliable (determined by each laboratory; typically 150–250 pg). Enhancing sensitivity of detection (34 cycles instead of 28 cycles). Too few copies of DNA template to ensure reliable PCR amplification. *Disadvantages*: Allele dropout, allele drop-in contamination, increased stutter, heterozygote imbalance, no thresholds, tissue source cannot be determined, DNA may not be relevant—casual contact/transfer—rarely useful for database searches.

1.20 Markers in Silkworm, *Bombyx mori*

The silkworm genomic analysis programs on identification of DNA markers for QTLs have been taken up by many researchers (Nagaraju and Goldsmith 2002; Rao and Chandrashekharaiah 2003; Reddy et al. 1999). Many workers have analyzed silkworm genome by ESTs (Kadono-Okuda et al. 2002) and SNPs (Yamamoto et al. 2006; Chatterjee and Mohandas 2003) and have identified eight molecular RFLP markers of which six are linked to high shell ratio and two to low cocoon shell ratio. Prudhome and Couble (2002) have succeeded in incorporating DNA sequence into silkworm genome using PiggyBAC technology.

A cDNA linkage map of silkworm, *Bombyx mori*, based on RFLP was constructed by Nguu et al. (2005) with RF02 female and F1 (RF02xRF50) male populations. Map consists of more than 194 randomly isolated cDNA clones whose

linkage groups which had been determined previously were analyzed, out of which 189 were unambiguously placed on the linkage map by repeated three-point analysis. The majority of the mapped clones corresponded to single loci dispersed on every 28 chromosomes. The map covers about 66% of the silkworm genome.

A RAPD linkage map of *Bombyx mori* was constructed by Li et al. (2000) with Dazao/C108 and their F2 generation. The map consists of 182 RAPD loci, of which 103 loci come from Dazao and from the first 23 linkage groups and the other 79 loci come from C108 and from the second 16 linkage groups. This map covered a total genetic distance of over 1148.3 cM.

Mita et al. (2004) established draft sequence of silkworm *Bombyx mori* by three-fold whole-genome shotgun (WGS) sequencing and assembled into 49,345 scaffolds that span a total length of 514 mb including gaps and 387 mb without gaps. Because the genome size of the silkworm is estimated to be 530 mb, almost 97% of the genome has been organized in scaffolds, of which 75% has been sequenced.

Yamamoto et al. (2006) have developed a linkage map for the silkworm *Bombyx mori* based on single nucleotide polymorphisms (SNPs) between strains p50T and C108T initially found on regions corresponding to the end sequences of bacterial artificial chromosome (BAC) clones. Using 190 segregants from a backcross of a p50T female × F1 (p50T × C108T) male, they analyzed segregation patterns of 534 SNPs, detected among 3840 PCR amplicons, each associated with a p50T BAC end sequence. They have constructed a linkage map composed of 534 SNP markers spanning 1305 cM in total length distributed over the expected 28 linkage groups.

Nagaraja and Nagaraju (1995) studied DNA profiling of 13 silkworm genotypes using the RAPD technique. Two hundred sixteen amplified products were generated using 40 random primers. Amplification products specific to diapausing genotypes were identified.

Nagaraja et al. (2005) also constructed a genetic map of RAPD, SSR, and FISSR markers for the Z chromosome using a backcross mapping population. Sixteen Z-linked markers were identified, characterized, and mapped using od, a recessive trait for translucent skin as an anchor marker yielding a total recombination map of 334.5 cM distributed throughout the Z chromosome. Four RAPD and four SSR markers that were linked to W chromosome were also identified.

Nagaraju et al. (2002) showed that the FISSR-PCR markers are inherited and segregated in Mendelian fashion as demonstrated on a panel of 99 F2 offspring derived from a cross of two divergent silkworm strains.

SilkDB 2017 (Silkworm Knowledgebase from China) (http://silkworm.genomics.org.cn) (Xia et al. 2004), SilkSatDB (SilkSatDB 2017) (a microsatellite database of silkworm from CDFD, India) (www.cdfd.org.in/silksatdb), Silkbase (Silkbase 2017) (EST database and BAC library from Japan) (www.ab.a.u-tokyo.ac.jp/silkbase) (Mita et al. 2004; Mita et al. 2003), and many other websites provide updated information about genome sequence assembly, cDNAs, ESTs, SNPs, and functional annotations of genes of silkworm.

References

Alwine JC, Kemp DJ, Stark GR (1977) Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. Proc Natl Acad Sci U S A 74(12):5350–5354

Anderson S (1981) Shotgun DNA sequencing using cloned DNase I-generated fragments. Nucleic Acids Res 9(13):3015–3027

Babak G, Jonas E, Nader N, Tommy N, Nyren P (2004) Improvements in pyrosequencing technology by employing sequence polymerase. Anal Biochem 330(2):272–280

Beavis WD, Grant D, Albertsen MC, Fincher RR (1991) Quantitative trait loci for plant height in four maize populations and their associations with qualitative genetic loci. Theor Appl Genet 83:141–145

Bornet B, Branchard M (2001) Nonanchored inter simple sequence repeat (ISSR) markers: reproducible and specific tools for genome fingerprinting. Plant Mol Biol Report 19:209–215

Burnette WN (1981) Western blotting: electrophoretic transfer of proteins from sodium dodecyl sulfate—polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Ann Biochem 112(2):195–203

Chatterjee SN, Mohandas TP (2003) Identification of ISSR markers associated with productivity traits in silkworm, *Bombyx mori* L. Genome 46(3):438–447

Edwards A, Caskey T (1991) Closure strategies for random DNA sequencing. Methods: Companion Methods Enzymol 3(1):41–47

Edwards A, Voss H, Rice P, Civitello A, Stegemann J, Schwager C, Zimmerman J, Erfle H, Caskey T, Ansorge W (1990) Automated DNA sequencing of the human HPRT locus. Genomics 6:593–608

Findlay I, Taylor A, Quirke P, Frazier R, Urquhart A (1997) DNA fingerprinting from single cells. Nature 389(6651):555–556

Fleischmann RD (1995) Whole-genome random sequencing and assembly of Haemophilus influenza. Science 269(5223):496–512

Gage GL (1974) Polyploidization of the silk gland of *Bombyx mori*. Chromosoma 45:27–42

Ghedin E, Bringaud F, Peterson J, Myler P, Berriman M, Ivens A, Andersson B, Bontempi E, Eisen J, Angiuoli S, Wanless D, Von Arx A, Murphy L, Lennard N, Salzberg S, Adams MD, White O, Hall N, Stuart K, Fraser CM, El-Sayed NM (2004) Gene synteny and evolution of genome architecture in trypanosomatids. Mol Biochem Parasitol 134(2):183–191

Goldsmith MR (1995) In: Goldsmith MR, Wilkins AS (eds) Molecular model system in the Lepidoptera. Cambridge University Press, New York, pp 21–76

Goldsmith MR (2006) What should we do with our molecular linkage maps? A position paper on positional cloning targets for the silkworm, *Bombyx mori*. In: Abstracts of Int. Symp. on Genetics and Genomics, CDFD, Hyderabad, India 9–11 Jan 2006

Goldsmith MR, Shimada T, Abe H (2005) The genetics and genomics of the silkworm, *Bombyx mori*. Annu Rev Entomol 50:71–100

Gupta M, Chyi Y-S, Romero SJ, Owen JL (1994) Amplification of DNA markers from evolutionarily diverse genomes using single primers of simple-sequence repeats. Theor Appl Genet 89:998–1006

Jeffreys AJ, McLead A, Tamaki K, Neil DL, Monckton DG (1991) Miniature repeat coding as a digital approach to DNA typing. Nature 354:204–209

Kadono-Okuda K, Kosegawa E, Mase K, Hara W (2002) Linkage analysis of maternal EST, cDNA clones covering all twenty-eight chromosomes in the silkworm, *Bombyx mori*. Insect Mol Biol 11(5):443–451

Kuribayashi S (1992) Sericulture technology. Farming Jpn 26(5):18–24

Li B, Lu C, Zhou ZY, Xiang ZH (2000) Construction of silkworm RAPD molecular linkage map. Yichuan Xuebao 27(2):27–32

Maxam AM, Gilbert W (1977) A new method for sequencing DNA. Proc Natl Acad Sci U S A 74(2):560–564

Mita K, Morimyo M, Okano K, Koike Y, Nahata J, Kawasaki H, Kadono-Okuda K, Yamamoto K, Suzuki MG, Shimada T, Goldsmith MR, Maeda S (2003) The construction of an EST database for *Bombyx mori*, and its application. Proc Natl Acad Sci U S A 100(24):4121–14126

Mita K, Kasahara M, Sasaki S, Nagayasu Y, Yamada T, Kanamori H, Namiki N, Kitagawa M, Yamashita H, Yasukochi Y, Kadono-Okuda K, Yamamoto K, Ajimura M, Ravikumar G, Shimomura M, Nagamura Y, Shin-I T, Abe H, Shimada T, Morishita S, Sasaki T (2004) The Genome Sequence of Silkworm, *Bombyx mori*. DNA Res 11(1):27–35

Mullis K (1998) Dancing naked in the mind field. Pantheon Books, New York

Nagaraja GM, Nagaraju J (1995) Genome fingerprinting of the silkworm, *Bombyx mori*, using random arbitrary primers. Electrophoresis 16(1):1633–1638

Nagaraja GM, Mahesh G, Satish V, Madhu M, Muthulakshmi M, Nagaraju J (2005) Genetic mapping of Z chromosome and identification of W chromosome-specific markers in the silkworm, *Bombyx mori*. Heredity 95:148–157

Nagaraju J, Goldsmith MR (2002) Silkworm genomics-progress and prospects. Curr Sci 83(4):415–425

Nagaraju J, Kathirvel M, Subbaiah EV, Muthulakshmi M, Kumar LD (2002) FISSR-PCR, A simple and sensitive assay for high throughput genotyping and genetic mapping. Mol Cell Probes 16:67–72

Nguu EK, Okuda KK, Mase K, Kosegawa E, Hara W (2005) Molecular linkage map for the silkworm, *Bombyx mori*, based on restriction fragment length polymorphism of cDNA clones. J Insect Biotechnol Sericol 74(1):5–13

van Oorschot RA, Treadwell S, Beaurepaire J, Holding NL, Mitchell RJ (2005) Beware of the possibility of fingerprinting techniques transferring DNA. J Forensic Sci 50(6):1417–1422

Peters P (1993) Biotechnology, A Guide to Genetic Engineering. WmC Brown, Dubuque

Prudhome JC, Couble P (2002) Perspectives in silkworm (*Bombyx mori*) transgenesis. Curr Sci 83(4):423–438

Rao CGP, Chandrashekharaiah M (2003) Molecular Marker Assisted Breeding in silkworm. Concept paper for Mulberry Silkworm Breeders Summit, APSSRDI, Hindupur, 18–19, July 2003

Reddy KD, Abraham EG, Nagaraju J (1999) Microsatellite in the silkworm, *Bombyx mori*: abundance, polymorphism and strain characterization. Genome 42(6):1057–1065

Renart J, Reiser J, Stark GR (1979) Transfer of proteins from gels to diazobenzyloxymethyl-paper and detection with antisera: a method for studying antibody specificity and antigen structure. Proc Natl Acad Sci U S A 76(7):3116–3120

Roach JC, Boysen C, Wang K, Hood L (1995) Pairwise end sequencing: a unified approach to genomic mapping and sequencing. Genomics 26:345–353

Ronald AH, van Oorschot RA, Jones MK (1997) DNA fingerprints from fingerprints. Nature 387(6635):767

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci U S A 74(12):5463–5467

SilkDB (2017) Silkworm knowledge base website from China (http://silkworm.genomics.org.cn)

Silkbase (2017) EST database and BAC library from Japan (www.ab.a.u-tokyo.ac.Jp/silkbase)

SilkSatDB (2017) A microsatellite database of silkworm website from CDFD, India (www.cdfd.org.in/silksatdb)

Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503–517

Staden R (1979) A strategy of DNA sequencing employing computer programs. Nucleic Acids Res 7:2601–2610

Tautz D (1989) Hypervariability of simple sequences as a general source for polymorphic DNA markers. Nucleic Acids Res 17(16):6463–6471

Tazima Y (1962) The genetics of silkworm. Logos Press, Academic Press, London, p p253

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A 76(9):4350–4354

Vos P, Hogers R, Bleeker M, Reijans M, Van-de-Lee T, Homes M, Frijters A, Pot J, Poleman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 23(21):4407–4414

Wang J, Xia Q, He X, Dai M, Ruan J, Chen J, Yu G, Yuan H, Hu Y, Li R, Feng T, Ye C, Lu C, Wang J, Li S, Wong GKS, Yang H, Wang J, Xiang Z, Zhou Z, Yu J (2005) SilkDB: a knowledgebase for silkworm biology and genomics. Nucleic Acids Res 33(Database issue):D399–D402

Watson JD (2007) Recombinant DNA: genes and genomes: a short course. San Francisco: W.H. Freeman. ISBN 0-7167-2866-4

White O (1995) In: Waterman MS (ed) Introduction to computational biology: sequences, maps and genomes. Boca Raton, CRC Press

Wikimedia (2003) https://commons.wikimedia.org/wiki/File%3ADNA_sequence.svg

Williums JGK, Kubelik AR, Livak KJ, Rafalaki JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 18:6231–6235

Wolfe AD, Liston A (1998) Contributions of PCR-based methods to plant systematics and evolutionary biology. In: Soltis DE, Soltis PS, Doyle JJ (eds) Plant molecular systematics-II. Kluwer, New York, pp 43–86

Wolfe AD, Xiang Q-Y, Kephart SR (1998) Assessing hybridization in natural populations of Penstemon (Scrophulariaceae) using hypervariable inter simple sequence repeat markers. Mol Ecol 7:1107–1125

Wu C, Kawasaki S, Yasukochi Y (1999) Construction and characterization of bacterial artificial chromosome libraries from the silkworm, *Bombyx mori*. Mol Gen Genet 261:698–706

Xia Q, Zhou Z, Lu C, Cheng D, Dai F, Li B et al (2004) A draft sequence for the genome of the domesticated silkworm (*Bombyx mori*). Science 306:1937–1940

Yamamoto K, Narukawa J, Kadono-Okuda K, Nohata J, Sasanuma M, Suetsugu Y, Banno Y, Fujii H, Goldsmith MR, Kazuei Mita K (2006) Construction of a single nucleotide polymorphism linkage map for the silkworm, *Bombyx mori*, based on bacterial artificial chromosome end sequences. Genetics 173(1):151–161

Yasukochi Y (1998) A dense genetic map of silkworm, *Bombyx mori,* covering all chromosomes based on 1018 molecular markers. Genetics 150:1513–1525

Yokoyama T (1973) The history of sericultural science in relation to industry. In: Smith RF, Mitter TE (eds) History of entomology. Annual Reviews, Palo Alto, pp 267–287

Zabeau M, Vos P (1993) Selective restriction fragment amplification: a general method for DNA fingerprinting. European Patent Office Publication 0534 858 A1, Bulletin 93/13

Zietkiewicz E, Rafalski A, Labuda D (1994) Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics 20:176–183

Molecular, Neuronal, and Behavioral Mechanism of Communication Among Insect Species: A Review

2

Iswar Baitharu, Sabita Shroff, and Jayanta Kumar Sahu

Abstract

Insects are the largest group of invertebrates having unique modalities of communication among members of the same species. Conspecific communication among insect species occurs mainly through visual, tactile, chemical, and behavioral changes. A number of studies on different insect models have been conducted by several researchers to understand the molecular, neuronal, and behavioral mechanism underlying communication among conspecifics. Though huge volume of research has been done to understand the mechanistic details of insect communication, there are a number of answered questions which require special attention. Understanding mechanisms of communication among insects has a number of potential applications in devising appropriate and sustainable control and/or management of insect population in the crop field. Pheromones are being used to effectively manage insect population since long before. Genetic basis of odor detections and interpretation of different odorants by insect species that carry message for different purposes involves several signaling receptors including G-protein-coupled receptor (GPCR) and second messenger signaling. Neuronal firing pattern following exposure to a pheromonal compound explains partially the mechanism of conspecific message delivery conspecific. However, how limited number of odorant-binding proteins that detect large spectrum of odorant species and differentiate as a different signal is not yet understood.

I. Baitharu (✉)
Department Environmental Sciences, Sambalpur University,
Jyoti Vihar, Burla 768019, Odisha, India
e-mail: iswarbaitharu@suniv.ac.in

S. Shroff
School of Chemistry, Sambalpur University, Jyoti Vihar, Burla 768019, Odisha, India

J.K. Sahu
Department of Life Science, National Institute of Technology, Rourkela, Odisha, India

D. Kumar, C. Gong (eds.), *Trends in Insect Molecular Biology and Biotechnology*, https://doi.org/10.1007/978-3-319-61343-7_2

2.1 Introduction

Communication is the process of exchange of information between two or more individuals of same or different species in which one individual transmits the message and the other receives the message, processes it, and gives appropriate response. In case of human being, communication ability is a result of a long learning process, while the same process appears to be an innate mechanism in insects. Individuals of insect species are usually born with a set of specific vocabulary which is shared only with the individuals of its own species. Communication can be an act of any part of an organism that invokes an alteration in the behavior of another organism as a response. Some emitter insects send a message using an acoustic signal to the rest of organisms by doing some action while some other insect species may do the same by developing certain physical traits such as the color pattern of wings of some butterflies. Reception of information from the emitter insect by other individual of same species occurs due to induction of some change in their receptor.

Similar to all other organisms, insects acquire information about their environment by using their five senses and exchange information among individuals of same species or other species. Some of these communication modalities may implicate contact senses such as taste and touch while other modalities may involve remote sense. Exchange of information using contact sense can occur only when two individuals come in direct physical contact with one another. Vision, olfaction, and auditory senses are the remote senses which are used frequently to promulgate information through the air or water over considerable distances. Thus, an insect may send a communication signal by making a noise, releasing a chemical, or flash a light, or the signal may simply be an inherent part of the insect's physical makeup such as wing pattern, body color, or surface chemistry. In either case, the signal must elicit some behavioral change in responding organism.

2.2 Necessity of Communication

There are a number of interesting social insects which lead a group life with distinct division of labor among them. Close coordination among the members of such insect species is essential at different level for various purposes such as reproduction, search for food sources. It is well known that members of an insect species communicate frequently with organisms of the same species which is referred to as intraspecific communication. Sometimes direct or indirect communication occurs between members of one species with organisms of other species for different purposes which is referred to as interspecific communication. There are a number of reasons for communication among insect species which are enumerated as follows:

- To search a courtship mate of same species for reproduction
- To identify members of the same species or even to warn other organisms of its own presence

- To convey information about the location of resources like food, nidification places, etc.
- As an alert signal toward potential hazards to make other individuals aware of it
- To protect territory for available resources in an area
- As a way to camouflage or to mimic other organisms (as a defensive strategy against predators)

2.3 Types of Communications Among Insect Species

2.3.1 Visual Communication

Communication using visual signals is common among different insect species. A number of different types of visual communications are displayed by various insect species such as the color patterns and other markings on the wings of butterflies and moths. The red admiral butterfly, for instance, possesses bright, typical markings on the upper wing surface and protective coloration on the underside. Some insect species communicate by emitting light of different intensities. For example, Lampyridae (beetle Order: Coleoptera) communicate using light with individual of its own species. Another good example of use of light as communication modality is seen among fireflies. Fire flies emit pulses of light as a courtship dialogue between a male which is usually flying and a female usually perched in the vegetation. Flash pattern of emitted light and response time to light are usually unique to a species of insect. Some insects possess unique capability of communication using wavelength in the ultraviolet light. Female cabbage butterflies is one of the best examples of it which have ultraviolet reflecting scales on the dorsal wing surface. When the female cabbage butterflies fly, each down stroke of the wing creates a brief flash of UV that males of the same species can recognize for mating.

2.3.2 Tactile and Behavioral Communication

Despite inevitable limitation of interaction between two or just a few individuals, tactile contacts form an important modality of communication among insect species. Because of their poor vision and sound receptor, numerous insect species mostly rely on physical contact for exchange of information. The antennation between nest mates is one of the common examples of tactile communication found in insect species. The inter-individual exchange of liquid material via trophallaxis is largely based on tactile interactions with mainly the antennae and the forelegs. Both ants and termites use antennal tapping as an essential component of tactile communication though exact information exchanged in such process is still not clear. However, nest mate recognition and exchange of food through trophallaxis could be the major result of antennal tapping. Insects touch each other's feelers to exchange messages. In case of blister beetles (Family: Meloidae) courtship usually begins

with a series of antennal taps by the male on each side of the female's body and male gets reciprocation from the female partner by lifting of wing covers and allowing to clump on the back.

Certain tree hoppers belonging to membracidae family produce vibrations in the tissue of their host plant which can be felt by all other tree hoppers residing on the same plant. Communication among bees exhibits a unique behavior similar to dance. Bees perform various types of dance to communicate the distance and direction of food sources as well as nest sites. Running in a circle popularly known as round dance is performed to indicate close sites and transitional or sickle dance for sites at an intermediate distance from the hive. This dance involves running in a semicircular or moon shape. The most complex of the dance types performed by honeybees is the waggle dance which generally performed by honey bee *Apis mellifera* to communicate the locations of food sources. The dance language of honey bee consists of different patterns that convey information about distance of food source from the bee hive. The number of interactions of the dance that bee performs conveys information about distance while the liveliness of dance indicates the quality of the food source. The angle of the dance provides information about the direction of the food source to other insects. Sometimes bees stop dancing and provide a food sample to other bees in the hive upon their request. Sound produced by bee during dance generally plays important role in getting attention of other bees and to keep their attention.

2.3.3 Acoustic Communication

Many insects have ability to produce sound though they possess no vocal chords. Insects use various other ways to produce sounds. Ways of producing sound include rubbing of body parts together. Sounds are caused by vibrations that can pass through air, water, and solid structures which insect use as a modality to convey various messages to the members of the same species or different species. Crickets sing by rubbing one wing over the other wing. Some other insects rub their legs, scratch their bodies, or rub their jaws together to make audible sound. Buzzing sound is produced by grasshoppers by rubbing the hind legs against the wings. Sound of different frequencies is produced by mosquito's resonation of antennal hairs. Special organs are also found in different insect species to produce sound. Male cicadas have special organs to produce sound called tymbals. Membranes present inside the tymbal can vibrate to produce a "singing" sound. A tympanic membrane in the abdomen (e.g., grasshoppers and moths) or in the tibiae of the front legs (e.g., crickets and katydids) is mostly used to detect sound. Though sound produced by most of the insects is clearly audible to human being such as that crickets' song, many insects make supersonic sounds that are above the human range of hearing. These supersonic sounds produced by insects have more than 20,000 vibrations each second. Some grasshoppers and moths have been known to produce ultrasonic sounds of 80,000 Hz.

2.3.4 Chemical Communication

One of the most common way of communication among insect species is the use of odor or smell. Special scent glands are present in insects that release small volatile odorant molecule from their body. These odors are popularly called as pheromones. The female insects can produce specific odorant molecules to attract partners of its own species for mating and such molecules are known as sex pheromones. Some insect species have extraordinary sensitivity toward the sex pheromone which they can perceive even at long distance. Male moths can perceive the pheromones of female moths over distances of many kilometers. Ants use odorant molecule to mark a trail, so that other ants can use the trail to get back to the nest or to find food. The special scent released by ants enables them to know the other members of their colony. Some insects use smell to notify about the danger to each other. Sense of taste or smell is sometimes exploited various insect species to detect the presence of odors. However, most insects possess specialized receptors in their feet, antennae, and ovipositors for perception of odorant signals. One of the most important organs for detecting odors in the insect species is the antennae. In species where the female produces an odor, the males often have extra big antennae which help them to find the female and the vice versa. These chemicals are divided into two groups:

1. *Pheromones*: These are low molecular weight volatile organic compounds released from specialized gland in insect species. Pheromones act as a chemical signals and it carries information from one member of a species to another member of the same species. The pheromones play crucial role in insect communication mostly as sex attractants, alarm substance, and many other intraspecific messages.
2. *Allelochemicals*: The chemical messages that are transmitted from an individual of one species to member of a different species occur through allelochemicals. These primarily include defensive signals such as repellents, compounds used to locate suitable host plant, and other signals to regulate interspecific behaviors.

2.4 Insect Hydrocarbons and Chemical Communication

A number of insect species use cuticular lipids, especially the hydrocarbons as a medium of chemical communication among themselves (Blomquist et al. 1998). Hydrocarbons released from insect species have been known to perform variety of functions such as sex attractants and aphrodisiacs, anti-aphrodisiacs, species, caste and kin recognition cues, aggregation pheromones, and kairomones. Insect chemoreceptors can distinguish hydrocarbons by the number and placement of methyl-branching groups, degree and positions of double bonds, and chain length. Apart from its role in communication, insect cuticular lipids also play crucial role

in restricting water loss and prevent a lethal rate of desiccation (Nelson and Blomquist 1995). It is challenging for all terrestrial animals with high surface area to volume ratio such as insects to conserve water in their bodies. The cuticular waxes function as anti-desiccation agent and play crucial role in meeting the need of water conservation and thus cuticular lipid is the focused target for insect control.

2.5 Neuronal Basis of Insect Communication

Insect species are known to communicate by secreting myriads of different volatile odorous compounds detection of which are encoded neurologically by the firing patterns of Olfactory Receptor Neurons (ORNs). The differential firing patterns of ORNs on exposure to compounds simultaneously determine the odor quality (chemical type), intensity (concentration), and dynamics (fluctuations in response time). The firing patterns of ORNs can be measured by in vivo recording from either population of ORNs or individual ORNs to study peripheral olfactory perception and odor coding. A number of studies show that recordings of ORN action potentials in response to odors are comprised of a limited number of discrete functional classes. Individual classes of ORNs can exhibit a diverse array of response properties to different odors in addition to their ability to respond differently to different sets of odors. Most neurons responding to odors are excitatory in nature but some neurons are also inhibited by certain odors. Various odor-specific onset and termination of kinetics of responses have also been displayed by ORNs. The cellular basis for an olfactory code is provided by different response spectra of the ORN types and their diverse response dynamics. Studies on response spectra of Drosophila ORNs suggest that during encoding of odors in insects, a single ORN can respond to multiple odorants and a single odorant can stimulate multiple ORN classes in a combinatorial way similar to that of mammals. Molecular studies at genetic level indicate that the distinct groups of genes encoding odorant receptor proteins are the underlying players of these responses. The OR (odorant receptor) genes underlying the responses of most of the ORN classes are now well characterized. While specialized ORNs clustered together within the main olfactory epithelium of the nasal cavity or the vomeronasal organ detect odorants in vertebrates, insect ORNs and their support cells remain in distinct olfactory sensilla on both antennal and maxillary palp structures (Stocker 1994). However, neuropil structures of the central nervous system (CNS) that participate in the synaptic relay of ORNs, glomeruli, are anatomically similar in both insect and vertebrate. Approximately 1000 in case of rat to 5000 number of glomeruli in case of dog are found in vertebrate's olfactory bulb (OB) (Hildebrand and Shepherd 1997). However, the antennal ORN axons project either ipsilaterally or bilaterally to number of glomeruli which ranges from 20 in *Aedes aegypti* (Anton 1996) to approximately 300 in a crustacean olfactory system in case of arthropods (Blaustein et al. 1993; Stocker 1994).

2.6 Molecular and Biochemical Basis of Chemical Communication

The molecular and biochemical basis of chemical communication in insect began to be understood when a broad class of water-soluble proteins was discovered in olfactory mucosa and sensilla which later on was found to be playing vital role in the olfactory process. These water-soluble proteins are secretory in nature and are known as Odorant Binding Proteins (OBPs). The initial identification and characterization of OBPs was based on its ability to directly bind known odorants in both insect (Vogt 1987) and vertebrate (Pevsner et al. 1985) systems. OBPs have been hypothesized to facilitate the solubilization of hydrophobic odorants molecules, act as its carrier, and elevate its effective concentration for receptor binding. Insect species are now known to possess a subset of OBPs with remarkable ability to bind with pheromone known as pheromone-binding proteins (PBPs) which are expressed in male-specific, pheromone-sensitive hairs (Vogt 1987). Similar proteins are also found in both male and female moths antenna structures but are associated with general odorant-sensitive neurons and hence are designated as general odorant-binding proteins (GOBPs) (Vogt et al. 1991). Large and diverse OBP/PBP family of olfactory proteins have now been identified in vertebrates as well as in various insect species including *A. mellifera*, *Drosophila melanogaster* (Pikielny et al. 1994) and true bugs (Dickens et al. 1998). However, there are numerous conflicting reports on physiological roles played by OBPs as some OBPs bind to a broad array of ligands with no visible specificity whereas some other OBPs have tremendous specificity in recognizing and binding only one class of odorant species (Dear et al. 1991).

The physiological function of odorant-binding proteins (OBPs) that mediate chemoreception in insects still poses number of unanswered questions. Studies show that the OBPs plays pivotal role in the overall process of olfactory signal transduction and slight change at genetic level can drastically effect the signaling process involving odorants. Kim et al. (1998) demonstrated that mutations in one candidate OBP gene, lush, resulted in defective ethanol sensitivity in *D. melanogaster*. It has been observed in fire ant *Solenopsis invicta* that OBP family proteins play crucial role in regulation of complex social behaviors (Krieger and Ross 2002). Further research on OBP family proteins is warranted to reveal the importance of these highly expressed olfactory proteins in numerous other species. One of the attractive hypotheses suggests that OBPs not only serve as a shuttle proteins responsible for bringing odorant ligands in proximity to olfactory receptors, OBPs could also play important role in increasing the complexity of olfactory inventory as a result of their differential affinity for particular odorants. Thus, the multiplicative binding affinities of both ORs and OBPs could represent the diverse olfactory sensitivity of an insect.

In addition to ligand based activation of receptor, cessation or reduction of signaling in response to repeated or persistent stimuli is an important component of sensory perception known as desensitization. Desensitization is observed in all

chemosensory systems in almost all organisms and can vary from complete termination of signaling to graded attenuation of agonist potency (Dohlman et al. 1991). Desensitization of GPCR-mediated signal transduction is carried out mainly through the combined activity of two classes of proteins: G-protein-coupled serine/threonine receptor kinases (GRKs) and arrestins (Freedman and Lefkowitz 1996). Second messenger-induced kinases such as cAMP-dependent protein kinase A (PKA) and protein kinase C (PKC) cause phosphorylation of specific intracellular residues on GPCRs resulting in slow desensitization, GRKs phosphorylate only the agonist-bound (activated) form of GPCRs and are responsible for rapid receptor-specific desensitization (Inglese et al. 1993). Phosphorylation by GRKs serves to promote the binding of arrestin proteins, which further uncouple GPCRs from the G-protein-based signaling cascade (Pippig et al. 1993).

Furthermore, GRKs and arrestins are also intimately involved in GPCR internalization, an integral component of GPCR resensitization (Ferguson et al. 1996). Recent studies show that visual arrestins also function in olfactory signal transduction pathways in *D. melanogaster* and *Anopheles gambiae* (Merrill et al. 2002), while huge number of ORs and OBPs are present in both these insects, only three genes encode dual-functional arrestins which make them an attractive target for reducing the olfactory sensitivity of insects of medical and economic importance. GPCRs contain seven transmembrane spanning regions of 20–25 amino acids and are most prevalent superfamily of proteins currently known and are having more than 5000 members (Gether 2000; Strader et al. 1994). These proteins link ligands and downstream effectors by transmitting, amplifying, and integrating other cellular signals (Dohlman et al. 1991). ORs being a member of the GPCR superfamily are hypothesized to function through a signal transduction pathway similar to other GPCRs and with specific components unique to olfactory tissue, such as Golf (a Gs-like protein), adenylate cyclase III, and cAMP-gated channel (Pilpel et al. 1998).

2.7 Pheromones in Communication Among Insect Partners

Due to the small body size of insects, their ability to produce and perceive auditory and visual signals over large distances is limited (Greenfield 2002). Social communication in insects largely depends on chemo sensation through chemicals involved in communication known as semiochemicals. Semiochemicals can be grouped into two classes: allelochemicals and pheromones. While allelochemicals are chemicals produced and secreted by one species of organism that elicit a behavioral or physiological response in a member of the other species, pheromones are those that elicit a response in a member of a same species (Wyatt 2014). Understanding the mechanisms behind chemical communications in insects using recent advances in insect genomics, molecular genetics, and neuroanatomical techniques has been a major focus because of its potent and high impact application in disease control and agriculture.

Diverse classes of chemicals such as ketones, aldehydes, and fatty acids have been co-opted by several insect species to serve as pheromones over time through

evolution (Yew and Chung 2015). The original function of cuticular hydrocarbons (CHCs) was as anti-desiccants but now it serves a dual role in pheromone signaling (Chung and Carroll 2015). Intrinsic properties of pheromones such as volatility vary depending upon its chemical nature. Some pheromones are volatile compounds while some are nonvolatile such as cuticular hydrocarbons. To coup with such variable volatility of pheromones, insects have evolved sophisticated pheromone-sensing organs for volatile and nonvolatile chemicals. While olfactory receptors present in the antennae and maxillary palps detect volatile pheromones like ketones, contact chemosensory receptors distributed across the body of the insect are implicated in the detection of low-volatile or nonvolatile pheromones, such as long chain CHCs (Ferveur 2005; Aquiloni et al. 2015).

Pheromones have been widely investigated as a sex attractant to drive behaviors associated with mating. Diverse classes of insect mating pheromones have been identified in numerous insect species which are secreted and perceived species specifically. Lepidopteran (butterflies and moths) are known to release volatile pheromones primarily for long-distance sexual advertisement (Greenfield 2002). On the other hand, fruit flies exploit both high-volatile and low-volatile CHCs pheromones for complex courtship behaviors (Haberer et al. 2014). Insects exhibiting dual parental care secrete pheromones to recognize mating partners (Müller et al. 2003). Beetle females are the best example that recognize their mate via nonvolatile CHC pheromones using contact chemo sensation mechanism (Wang and Anderson 2010; Carde 2014). Male–male interactions like aggression are also regulated by pheromonal signaling in many insect species. For example, a male-specific volatile pheromone 11-cis-vaccenyl acetate (cVA) is secreted by *D. melanogaster* that pleiotropically suppresses male–male courtship and aggression (Wertheim et al. 2006).

Apart from mating and sexual behaviors, pheromones are also used as a signal to induce the formation of groups of conspecifics and designated as aggregation pheromones (Imen et al. 2015). Aggregation pheromones are typically volatile long distance signal and are perceived by the olfactory system (van Zweden and d'Ettorre 2010). However, the cockroach, *Periplaneta americana*, uses both high-volatile and low-volatile CHCs as a signal for aggregation at diurnal resting site (Suh et al. 2014). Additionally, pheromone-driven social behaviors such as nest mate recognition and nest defense are independent of mating and are prevalent in social insects. Volatile alarm pheromones are mostly used for recruiting conspecifics to attack intruders (Wyatt 2014; Sakurai et al. 2014).

Insect species come across large numbers of volatile organic compounds of natural as well as anthropogenic origin. Thus it is imperative for insects to differentiate a myriad of physiologically irrelevant chemical compounds in the environment from essential semiochemical signals such as sex pheromones. The ability of pheromones in conveying message to the conspecific insects is dependent on chemical structure of the molecule and even tiny change in the pheromone molecules renders them completely inactive (Kaissling 1987). The extraordinary selectivity of the olfactory system (i.e., its ability to discriminate) is coupled with an inordinate sensitivity. To advertise their readiness to mate for reproduction, females secrete very minute quantity of sex pheromones and thus avoid being noticeable. On the other hand, detectors

in males display remarkable sensitivity and perceive such small amounts of pheromone in a way that the signal-to-noise ratio of the system approaches the theoretical limit. Furthermore a dynamic process of signal inactivation is a prerequisite in case of odor oriented navigation. Males encounter pheromone molecules as flashing signals consisting of diminutive burst of high flux estranged by periods during which the flux is zero while flying toward a pheromone releasing female. The average duration of spikes within puffs of pheromones is on the millisecond scale, and it declines as the moth approaches the source of pheromone (Murlis et al. 2000). Thus, a male moth has to perceive selectively minute quantity of pheromones and reset the pheromone detectors on a millisecond timescale.

2.8 Molecular Mechanism of Odor Reception in Insects

Olfactory receptors (ORs) and odorant binding proteins (OBPs) have been studied extensively to understand their role in odor sensitivity and discrimination. OBPs have got special attention as regulator of dynamics of olfaction system in insects as well as in higher vertebrates which has been two strong line of evidence as below:

First, expression of a Drosophila odorant receptor in *Xenopus oocytes* provided direct evidence for its function, its activation was slower requiring timescale of second than normally observed millisecond timescale in in vivo function. This extreme slow response of ORs could be because of lack of OBPs in the heterologous system of xenopus olfaction process.

Second, kinetic studies demonstrated that the pH-dependent conformational change in BmPBP requires less than 4 ms. Studies on structural biology aspect of the molecules indicate that conformational change in BmPBP is an intramolecular mechanism to facilitate binding and release of pheromones by pheromone-binding proteins. Whether the remarkable selectivity of the insect's olfactory system (Kaissling 1987) is achieved by the specificity of pheromone-binding proteins or the olfactory receptors is still unclear. When tested with a limited number of candidate ligands, OBPs bind to candidate ligands specifically (Du and Prestwich 1995; Maïbèche-Coisné et al. 1997; Maida et al. 2000; Plettner et al. 2000; Wojtasek et al. 1999). However, the number of OBPs is significantly less than the number of compounds that insects can smell. Even in the case of Drosophila, a species which has been extensively studied, only a few number of OBPs have been identified (Graham and Davies 2002). How limited number of OBPs detect unlimited numbers of different odorant species is still a matter of research. Evidences show that a Drosophila olfactory receptors are not specific to a single ligand (Wetzel et al. 2001). It can be stimulated by compounds with remarkably different chemical structures, such as cyclohexanol and cyclohexanone, benzaldehyde, and benzyl alcohol. The extraordinary specificity of insect olfactory system has been extensively explored using pioneering electroantennogram (Schneider 1957) and single sensillum recordings (Schneider and Boeckh 1962) at the Max Planck Institute. Even the generalist detectors for plant compounds have now been demonstrated to have inordinate specificity (Hansson and Christensen 1999; Nikonov et al. 2001; Nikonov et al.

2002). The mechanism of such specificity of a receptor could be based on the concept of "layers of filters" of participating OBPs that operate step by step. OBPs transport only small subset of the ligands to reach the pore tubule where each OR can be stimulated by a small number of ligands out of which only few of them reach the dendrite. Thus though neither the OBPs nor the ORs are extremely specific, the whole machinery can show remarkable selectivity by acting as two step filter.

2.9 Pheromone Detection by Olfactory Systems

The antennae and maxillary palps are the primary sensory organs in insects that detect volatile ligands. A huge array of anatomically and functionally diverse specialized structure called sensilla cover these organs. Inside the sensilla, olfactory receptor neurons (ORNs) are found in large numbers that are responsible for the detection of various chemicals (Suh et al. 2014). For example, four different types of sensilla are found on the antennae of silkworm *Bombyx mori*, out of which three are found to detect general, non-pheromone chemicals while the other one a long trichodea is uniquely tuned for detection of the sex pheromones such as bombykol and bombykal (Sakurai et al. 2014). *D. melanogaster* possess a trichoid sensilla which can specifically detect volatile pheromones like cVA and methyl laurate (ML) (Dweck et al. 2015). Chemical and molecular identities of diverse compound acting as pheromones are well characterized, however, the receptors responsible for specifically detecting such diverse pheromones in insect species are still unexplored. Though the recent advancement in *Drosophila* molecular genetics and in some insects has largely filled this gap, neurophysiological processes and behavioral alteration involved in pheromonal signaling require further research in numerous other insect species. It is now known that two different families of olfactory receptors (ORs) seem to detect the majority of insect volatile pheromones Kaissling (1986). The members of the olfactory receptor family were identified first of all as volatile pheromone receptors (Vosshall et al. 2000; Clyne et al. 1999). cVA, a known pheromone, was shown to activate and inhibit innate behavioral programs via the activation of Or67d expressing and Or65a-expressing neurons using neuronal and behavioral approaches (Datta et al. 2008; Liu et al. 2011). Furthermore, these neuronal and genetic architectures have been known to be evolutionarily conserved across the Drosophila species group (Dweck et al. 2015; Dekker et al. 2015; Lebreton et al. 2014). Pheromone receptor neurons synapse with central projection neurons in discrete glomeruli within the antennal lobe similar to olfactory receptor neurons.

2.10 Behavioral Mechanism of Communication Among Insect Species

Most of the insects live a solitary life except few conspecific contacts. Temporary aggregations among the insect species is often associated with the abundance of food materials as in case of grasshoppers and the encounter of conspecific males

and females prior to copulation during breeding season. Social insects are characterized by the communities where they live in permanent association with their nest mates. In this regard, bees, bumblebees, wasps, ants, and termites have fascinated human beings due to their well-organized and impressive colonies. The social lifestyle of insects goes along with the foreseeable development of a communication system which allows the individual members of the colony to exchange information. This mode of communication occurs through various sensory channels, using visual, acoustic, tactile, sometimes magnetic, and especially chemical signals.

2.11 Honeybee Dancing

The best-known example of communication among social insect communication is the dancing event that is used by the honeybee workers to instruct their nest mates regarding the food sources. Karl von Frisch unraveled the significance of this dancing behavior and has been honored with Nobel Prize for this achievement (Frisch 1954; Frisch et al. 1967). Bee workers returning from a successful foraging journey enter the nest and perform peculiar dances on the vertical nest combs to communicate information about the food source to the nest mates. Subsequently, the nearby nest mates decipher the encoded information and recognize the distance and direction of the food source. This exchange of information in the form of waggle dance involves various stimuli such as visual cues, chemical cues. The returning forager bee gives visual cues to the nest mates about direction of the food source by orienting itself with respect to the position of the sun. The odor in the nest entrance that acts as a chemical cue helps the returning bees to recognize its own nest. Once the returning bee enters the bee hive, communication to the nest mate about the food sources depends mostly on the tactile cues through direct body contact to the nearby nest mate because of the darkness inside the bee hive. Through the direct body contact the dancing bee provides a chemical cue nest mates by offering some collected nectar so that they can recognize the target food source. The acoustic cues come from the buzzing sound of the dancer's moving wings that play essential role in conveying the exact position of the food source (Michelsen et al.1989). Subsequent movement of the nest mates from bee hive to the food source mostly relies upon the sun compass as a visual cue to localize the exact position of food source. However, the visual communication using visual cues is not common in social insects because of the fact that insects possess compound eye with poor vision. For visual tracking of the foraging leading bee to the food source, a well-developed sight is an utmost necessity which the insect species lack (Nieh 2004).

In some insect species, the big compound eyes of the males facilitate in localizing the females partner before mating and also for orientation during mating. Ants because of their exceptional visual capacities detect polarized light to orient themselves for different purposes (Wehner 2003). Wood ant also uses its visual capacity for recognition of environmental patterns. The foraging workers of the wood ant can reopen the same routes accurately which they had followed in last summer after hibernation. Change in the environmental cues such as felling of tress has been reported to

drastically decrease the fidelity of the reopened route (Rosengren and Pamilo 1978). However, some insect species possess no visual system such as eye and hence visual cues play no role in their case. For example, some ants and termite species because of total absence of eyes cannot use visual cues. Winged social insects use acoustic communication by producing buzzing sound through high-frequency wing movements. As in the case of honey bee, sound produced through rapid wing movement at high frequency and movement of thoracic muscle during waggle dance helps to attract attention and provide information about distance and quality of food sources to the nest mates (Nieh 2004). The queen's tooting and quacking signals give acoustic communication about newly enclosing queens to make contact with each other (Michelsen et al. 1986). Sounds produced by knocking body parts onto the substrate called as drumming in wingless termites (Röhrig et al. 1999) and in some ants provide acoustic signals and bring about behavioral responses (Hölldobler 1999).

Stridulation behavior in some ant species such as rapid movement of the scraper situated at the posterior dorsal margin against parallel ridges of first gastral tergite plays important role in nest mate selection. Atta ants stridulate while cutting leaf fragments in order to recruit nest mates (Roces and Hölldobler 1996; Eibl-Eibesfeldt and Eibl-Eibesfeldt 1967). Stridulation activities appear to regulate ant's species in maneuvering the leaf fragment into a carrying position (Roces and Hölldobler 1995). However, there are a number of controversial reports regarding the transmission of ant stridulatory signals through air (Hickling and Brown (2000). It is still unknown whether the ants are deaf and hence detection of sound occur through substrate-borne vibrations and not by sound produced (Roces and Tautz 2001).

2.12 Magnetic Orientation

Magnetic orientation among few insect species such as ants with respect to earth's magnetic field has been reported to be used as communication modalities. Several reports suggest that the magnetic nanoparticles present in the body of the insect detect the geomagnetic (Acosta-Avalos et al. 1999). In the absence of sunlight cues, leaf-cutting ants appear to be responding to the geomagnetic field during its foraging journey (Banks and Srygley 2003). The ability to perceive the earth's magnetic field has also been demonstrated in a number of insect species such as the fire ant *Solenopsis invicta* (Anderson and Vander Meer 1993), bees (Gould 1980), and bumble bees (Chittka et al. 1998). However, orientation along the earth's magnetic field is not a true mode of communication among the insect species.

Conclusion

Communication among insect species involves complex process of exchange of information encoded in semiochemicals like pheromones, sex attractants, acoustic exchange of messages through production of unique sound, complex and peculiar behavior, and highly sensitive and selective reception of signals. In spite of many years of research into the role of pheromones and other related factors regulating the behavior of insects, our understanding of the mechanisms and

evolutionary processes that support these complex signals is still in their infancy. Although studies in the fruit fly *D. melanogaster* are paving the way for understanding the sensory, neuroethological, and genetic principles of pheromonal communication, the current lack of comparable genetic tool for other insect species hinders progress in the field. A number of studies have been undertaken in the recent past to understand the neuronal, molecular, and behavioral basis of insect communication in few insect models. Insects are the members of largest phylum arthropoda with huge numbers of insect species and unique communication modalities. Insect pest is the major threat to modern crop system that includes numerous hybrid varieties with reduced pest resistance. Modern agricultural practice in recent years has introduced large numbers of dangerous persistent pesticides to the environment which has resulted in incidence of number of diseases in the human system. Understanding the mechanism of insect communication would help in managing pest species without polluting the environment. For that purpose, identification of receptors and cells responsible for pheromonal communication in diverse insect species will enable the field to take advantage of the wealth of existing behavioral and physiological data from these species. Furthermore, as a number of insect species act as pest or as disease vectors, understanding the mechanism of pheromonal signaling in regulating behavior of these insect species can be implicated for the development of more sustainable and specific environment-friendly control methods.

References

Acosta-Avalos D, Wajnberg E, Oliveira PS, Leal I, Farina M, Esquivel DMS (1999) Isolation of magnetic nanoparticles from *Pachycondyla marginata* ants. J Exp Biol 202:2687–2692

Anderson JB, Vander Meer RK (1993) Magnetic orientation in the fire ant, *Solenopsis invicta*. Naturwissenschaften 80:568–570

Anton S (1996) Central olfactory pathways in mosquitoes and other insects. Ciba Found Symp 200:184–192. discussion 192–196, 226–232

Aquiloni L, Tricarico E, SpringerLink (Online service) Springer International Publishing (2015) In: Aquiloni L, Tricarico E (eds) Social recognition in invertebrates the knowns and the unknowns. Springer International Publishing, Berlin

Banks AN, Srygley RB (2003) Orientation by magnetic field in leaf-cutter ants, *Atta Colombica* (hymenoptera: Formicidae). Ethology 109:835–846

Blaustein DN, Simmons RB, Burgess MF, Derby CD, Nishikawa M, Olson KS (1993) Ultrastructural localization of 5’AMP odorant receptor sites on the dendrites of olfactory receptor neurons of the spiny lobster. J Neurosci 13:2821–2828

Blomquist GJ, Tillman JA, Mpuru S, Seybold SJ (1998) The cuticle and cuticular hydrocarbons of insects: structure, function and biochemistry. In: Vander Meer RK, Breed M, Winston M, Espelie C (eds) Pheromone communication in social insects. Westview Press, Boulder, CO, pp 34–54

Carde RT (2014) Defining attraction and aggregation pheromones: teleological versus functional perspectives. J Chem Ecol 40:519–520

Chittka L, Williams NM, Rasmussen H, Thomson JD (1998) Navigation without vision: bumblebee orientation in complete darkness. Proc R Soc Lond B 266:45–50

Chung H, Carroll SB (2015) Wax, sex and the origin of species: dual roles of insect cuticular hydrocarbons in adaptation and mating. BioEssays 37:822–830

Clyne PJ, Warr CG, Freeman MR, Lessing D, Kim J, Carlson JR (1999) A novel family of divergent seven-transmembrane proteins: candidate odorant receptors in drosophila. Neuron 22:327–338

Datta SR, Vasconcelos ML, Ruta V, Luo S, Wong A, Demir E, Flores J, Balonze K, Dickson BJ, Axel R (2008) The drosophila pheromone cVA activates a sexually dimorphic neural circuit. Nature 452:473–477

Dear TN, Boehm T, Keverne EB, Rabbitts TH (1991) Novel genes for potential ligand-binding proteins in subregions of the olfactory mucosa. EMBO J 10:2813–2819

Dekker T, Revadi S, Mansourian S, Ramasamy S, Lebreton S, Becher PG, Angeli S, Rota-Stabelli O, Anfora G (2015) Loss of drosophila pheromone reverses its role in sexual communication in *Drosophila suzukii*. Proc Biol Sci 282:20143018

Dickens JC, Callahan FE, Wergin WP, Murphy CA, Vogt RG (1998) Odorant binding proteins of true bugs. Generic specificity, sexual dimorphism, and association with subsets of chemosensory sensilla. Ann N Y Acad Sci 855:306–310

Dohlman HG, Thorner J, Caron MG, Lefkowitz RJ (1991) Model systems for the study of seven-transmembrane-segment receptors. Annu Rev Biochem 60:653–688

Du G, Prestwich GD (1995) Protein structure encodes the ligand binding specificity in pheromone binding proteins. Biochemistry 34:8726–8732

Dweck HK, Ebrahim SA, Thoma M, Mohamed AA, Keesey IW, Trona F, Lavista-Llanos S, Svatos A, Sachse S, Knaden M et al (2015) Pheromones mediating copulation and attraction in drosophila. Proc Natl Acad Sci U S A 112:E2829–E2835

Eibl-Eibesfeldt I, Eibl-Eibesfeldt E (1967) Das Parasitenabwehren der Minima- Arbeiterinnen der Blattschneider-Ameise (*Atta cephalotes*). Z Tierpsychol 24:278–281

Ferguson SS, Downey WER, Colapietro AM, Barak LS, Menard L, Caron MG (1996) Role of beta-arrestin in mediating agonist-promoted G protein-coupled receptor internalization. Science 271:363–366

Ferveur JF (2005) Cuticular hydrocarbons: their evolution and roles in drosophila pheromonal communication. Behav Genet 35:279–295

Freedman NJ, Lefkowitz RJ (1996) Desensitization of G protein-coupled receptors. Recent Prog Horm Res 51:319–351. discussion 352–353

Frisch K (1954) The dancing bees: an account of the life and senses of the honey bee, 1st edn. Springer-Verlag, Wien, pp XIV–183

Frisch K, Wenner AM, Johnson DL (1967) Honeybees: do they use direction and distance information provided by their dancers? Science 158:1072–1077

Gether U (2000) Uncovering molecular mechanisms involved in activation of G protein coupled receptors. Endocr Rev 21:90–113

Gould JL (1980) The case for magnetic sensitivity in birds and bees (such as it is): surprising concentrations of magnetite in the tissues of some animals may explain their sensitivity to the earth's magnetic field. Am Sci 68:256–267

Graham LA, Davies PL (2002) The odorant-binding proteins of *Drosophila melanogaster*. Annotation and characterization of a divergent gene family. Gene 292:43–55

Greenfield MD (2002) Signalers and receivers: mechanisms and evolution of arthropod communication. Oxford University Press, Oxford

Haberer W, Steiger S, Müller JK (2014) Dynamic changes in volatile emissions of breeding burying beetles. Physiol Entomol 39:153–164

Hansson BS, Christensen TA (1999) Functional characteristics of the antennal lobe. In: Hansson BS (ed) Insect olfaction. Springer, Berlin, pp 125–161

Hickling R, Brown RL (2000) Analysis of acoustic communication by ants. J Acoust Soc Am 108:1920–1929

Hildebrand JG, Shepherd GM (1997) Mechanisms of olfactory discrimination: converging evidence for common principles across phyla. Annu Rev Neurosci 20:595–631

Hölldobler B (1999) Multimodal signals in ant communication. J Comp Physiol 184A:129–141

Imen S, Christian M, Virginie D, Colette R (2015) Intraspecific signals inducing aggregation in *Periplaneta americana* (Insecta: Dictyoptera). Environ Entomol 44:713–723
Inglese J, Freedman NJ, Koch WJ, Lefkowitz RJ (1993) Structure and mechanism of the G protein-coupled receptor kinases. J Biol Chem 268:23735–23738
Kaissling K-E (1986) Temporal characteristics of pheromone receptor cell responses in relation to orientation behaviour of moths. In: Payne TL, Birchm MC, Kennedy CEJ (eds) Mechanisms in insect olfaction. Oxford University Press, Oxford, pp 193–200
Kaissling K-E (1987) In: Colbow K (ed) R. H. Wright lectures on insect olfaction. Simon Fraser University, Burnaby, BC
Kim MS, Repp A, Smith DP (1998) LUSH odorant-binding protein mediates chemosensory responses to alcohols in *Drosophila melanogaster*. Genetics 150:711–721
Krieger MJ, Ross KG (2002) Identification of a major gene regulating complex social behavior. Science 295:328–232
Lebreton S, Grabe V, Omondi AB, Ignell R, Becher PG, Hansson BS, Sachse S, Witzgall P (2014) Love makes smell blind: mating suppresses pheromone attraction in drosophila females via Or65a olfactory neurons. Sci Rep 4:7119
Liu W, Liang X, Gong J, Yang Z, Zhang YH, Zhang JX, Rao Y (2011) Social regulation of aggression by pheromonal activation of Or65a olfactory neurons in drosophila. Nat Neurosci 14:896–902
Maïbèche-Coisné M, Sobrio F, Delaunay T, Lettere M, Dubroca J, Jacquin-Joly E, Nagnan-LeMeillour P (1997) Pheromone binding proteins of the moth mamestra brassicae: specificity of ligand binding. Insect Biochem Mol Biol 27:213–221
Maida R, Krieger J, Gebauer T, Lange U, Ziegelberger G (2000) Three pheromone binding proteins in olfactory sensilla of the two silkmoth species *Antheraea polyphemus* and *Antheraea Pernyi*. Eur J Biochem 267:2899–2908
Merrill CE, Riesgo-Escovar JR, Pitts RJ, Kafatos FC, Carlson JR, Zwiebel LJ (2002) Visual arrestins in olfactory pathways of drosophila and the malaria vector mosquito *Anopheles gambiae*. Proc Natl Acad Sci U S A 99:1633–1638
Michelsen A, Kirchner WH, Andersen BB, Lindauer M (1986) The tooting and quacking vibration signals of honeybee queens: a quantitative analysis. J Comp Physiol 158A:605–611
Michelsen A, Andersen BB, Kirchner WH, Lindauer M (1989) Honeybees can be recruited by a mechanical model of a dancing bee. Naturwissenschaften 76:277–280
Müller JK, Eggert A-K, Elsner T (2003) Nestmate recognition in burying beetles: the "breeder's badge" as a cue used by females to distinguish their mates from male intruders. Behav Ecol 14:212–220
Murlis J, Willis MA, Cardé RT (2000) Spatial and temporal structures of pheromone plumes in fields and forests. Physiol Entomol 25:211–222
Nelson DR, Blomquist GJ (1995) Insect waxes. In: Hamilton RJ, Christie WW (eds) Waxes: chemistry, molecular biology and functions. The Oily Press, England, pp 1–90
Nieh JC (2004) Recruitment communication in stingless bees (hymenoptera, Apidae, Meliponinae). Apidologie 35:159–182
Nikonov AA, Valiyaveettil JT, Leal WS (2001) A photoaffinity-labeled green leaf volatile compound "tricks" highly selective and sensitive insect olfactory receptor neurons. Chem Senses 26:49–54
Nikonov AA, Peng G, Tsurupa G, Leal WS (2002) Unisex pheromone detectors and pheromone-binding proteins in scarab beetles. Chem Senses 27:495–504
Pevsner J, Trifiletti R, Strittmatter SM, Synder SH (1985) Isolation and characterization of an olfactory receptor protein for odorant pyrazines. Proc Natl Acad Sci U S A 82:3050–3054
Pikielny CW, Hasan G, Rouyer F, Rosbash M (1994) Members of a family of drosophila putative odorant-binding proteins are expressed in different subsets of olfactory hairs. Neuron 12:35–49
Pilpel Y, Sosinsky A, Lancet D (1998) Molecular biology of olfactory receptors. Essays Biochem 33:93–104

Pippig S, Andexinger S, Daniel K, Puzicha M, Caron MG, Lefkowitz RJ, Lohse MJ (1993) Overexpression of beta-arrestin and beta-adrenergic receptor kinase augment desensitization of beta 2-adrenergic receptors. J Biol Chem 268:3201–3208
Plettner E, Lazar J, Prestwich EG, Prestwich GD (2000) Discrimination of pheromone enantiomers by two pheromone binding proteins from the gypsy moth *Lymantria dispar*. Biochemistry 39:8953–8962
Roces F, Hölldobler B (1995) Vibrational communication between hitchhikers and foragers in leaf-cutting ants (*Atta cephalotes*). Behav Ecol Sociobiol 37:297–302
Roces F, Hölldobler B (1996) Use of stridulation in foraging leaf-cutting ants: mechanical support during cutting or short-range recruitment signal? Behav Ecol Sociobiol 39:293–299
Roces F, Tautz J (2001) Ants are deaf. J Acoust Soc Am 109:3080–3082
Röhrig A, Kirchner WH, Leuthold RH (1999) Vibrational alarm communication in the African fungus-growing termite genus Macrotermes (Isoptera, Termitidae). Insect Soc 46:71–77
Rosengren R, Pamilo P (1978) Effect of winter timber felling on behaviour of foraging wood ants (Formica rufagroup) in early spring. Memorabilia Zool 29:143–155
Sakurai T, Namiki S, Kanzaki R (2014) Molecular and neural mechanisms of sex pheromone reception and processing in the silkmoth *Bombyx mori*. Front Physiol 5:125
Schneider D (1957) Elektrophysiologische untersuchungen von chemo- und mechanorezeptoren der antenne des seidenspinners *Bombyx mori* L. Z Vergl Physiol 40:8–41
Schneider D, Boeckh J (1962) Rezeptorpotential und nervenimpulse einzelner olfaktorischer sensillen der insektenantenne. Z Vergl Physiol 45:405–412
Stocker RF (1994) The organization of the chemosensory system in *Drosophila melanogaster*: a review. Cell Tissue Res 275:3–26
Strader CD, Fong TM, Tota MR, Underwood D (1994) Structure and function of G protein-coupled receptors. Annu Rev Biochem 63:101–132
Suh E, Bohbot JD, Zwiebel LJ (2014) Peripheral olfactory signaling in insects. Curr Opin Insect Sci 6:86–92
van Zweden JS, d'Ettorre P (2010) Nestmate recognition in social insects and the role of hydrocarbons. In: Insect hydrocarbons: biology, biochemistry and chemical ecology, vol 11. Cambridge University Press, Cambridge, pp 222–243
Vogt RG (1987) The molecular basis of pheromone reception: its influence on behavior. In: Prestwich GD, Blomquis GJ (eds) Pheromone biochemistry. Academic Press, New York, pp 385–431
Vogt RG, Rybczynski R, Lerner MR (1991) Molecular cloning and sequencing of general odorant-binding proteins GOBP1 and GOBP2 from the tobacco hawk moth *Manduca sexta*: comparison with other insect OBPs and their signal peptides. J Neurosci 11:2972–2984
Vosshall LB, Wong AM, Axel R (2000) An olfactory sensory map in the fly brain. Cell 102:147–159
Wang L, Anderson DJ (2010) Identification of an aggression-promoting pheromone and its receptor neurons in drosophila. Nature 463:227–231
Wehner R (2003) Desert ant navigation: how miniature brains solve complex tasks. J Comp Physiol 189:579–588
Wertheim B, Allemand R, Vet LEM, Dicke M (2006) Effects of aggregation pheromone on individual behaviour and food web interactions: a field study on drosophila. Ecol Entomol 31:216–226
Wetzel CH, Behrendt H-J, Gisselmann G, Störtkuhl KF, Hovemann B, Hatt H (2001) Functional expression and characterization of a drosophila odorant receptor in a heterologous cell system. Proc Natl Acad Sci 98:9377–9380
Wojtasek H, Picimbon JF, Leal WS (1999) Identification and cloning of odorant binding proteins from the scarab beetle *Phyllopertha diversa*. Biochem Biophys Res Commun 263:832–837
Wyatt TD (2014) Pheromones and animal behavior: chemical signals and signatures, 2nd edn. Cambridge University Press, Cambridge
Yew JY, Chung H (2015) Insect pheromones: an overview of function, form, and discovery. Prog Lipid Res 59:88–105

3

Monocyclic Aromatic Hydrocarbons (MAHs) Induced Toxicity in *Drosophila*: How Close How Far?

Mahendra P. Singh and Ranjana Himalian

Abstract

Monocyclic aromatic hydrocarbons (MAHs) are being used as individual chemicals or as in mixtures of two or more chemicals in several industrial and household processes across the world. Among MAHs, the most common chemicals are benzene, toluene and xylene, and they are also known as volatile organic compounds (VOCs), among them benzene categorised as highly toxic chemical and also listed as human carcinogen. Benzene, toluene and xylene cause cytotoxicity to a nontarget organism-like *Drosophila melanogaster* as an individual (benzene or toluene or xylene)/in mixture (benzene-toluene-xylene or benzene-toluene or benzene-xylene). In this chapter, several cellular, biochemical and molecular approaches were used to evaluate cellular toxicity due to MAHs like benzene, toluene and xylene using *Drosophila melanogaster* as an alternative to animal. We also judged variable cytotoxicity patterns of MAHs when they are exposed individually or in a mixture of two/three chemicals. An antagonistic effect of xylene and toluene on benzene toxicity and additive/synergistic effect of xylene on toluene-induced toxicity were evident in *Drosophila*. This study shows that co-exposure of benzene-toluene-xylene causes reduced cellular and organismal toxicity as compared to individual test chemical on *Drosophila melanogaster*.

M.P. Singh (✉) • R. Himalian
School of Bioengineering and Biosciences, Lovely Professional University,
Jalandhar-Delhi GT Road (NH-1), Phagwara 144411, Punjab, India
e-mail: mprataps01@gmail.com

D. Kumar, C. Gong (eds.), *Trends in Insect Molecular Biology and Biotechnology*, https://doi.org/10.1007/978-3-319-61343-7_3

3.1 Introduction

Monocyclic aromatic hydrocarbons have diverse ring and side chain structure that could independently affect their absorption and toxicity effectively (Chou et al. 2003). Unleaded petrol contains significant amount of benzene, toluene and xylene, collectively called as BTX. A number of chemicals are used in chemical and pharmaceutical industries. Among the major monocyclic aromatic hydrocarbons (MAHs) are styrene, benzene, toluene and isomers *o*-xylene, *m*-xylene and *p*-xylene. These chemicals are constituted in gasoline up to 15% and also significantly present in paints, plastics, detergents, dyes, adhesives, pesticides, rubber products and several other products (Derwent et al. 2000; Kim and Kim 2002; Chang et al. 2007). They are one of the major pollutants of the outdoor and indoor environment (Lee et al. 2002; Srivastava and Devotta 2007). Benzene is causally linked to leukaemia (IARC) (US-EPA 1996), whereas currently toluene and xylene are not listed as carcinogens (Gallegos et al. 2007). BTX is a special lipophilic substance that has capability to penetrate the skin or subcutaneous fat (Adami et al. 2006a, b and also used as an individual or as a mixture compound. It is shown that benzene is more toxic as compared to toluene and xylene (Singh et al. 2009, 2010, 2011). Earlier studies reported the presence of benzene and benzene mixtures in different occupational environments, like leather, electronics, machinery and sports equipment industries (Wong and Raabe 1989; Dosemeci et al. 1994; Kuang and Liang 2005; Wang et al. 2006). Exposure to these nonoxygenated aromatic hydrocarbons is of great concern, even at low concentrations because of their toxicity and carcinogenicity. An increasing number of young people inhale these volatile substances for recreational purposes (Greer 1984; Kozel et al. 1995; Spiller and Krenzelok 1997).

Toluene has a protective effect on male mice against benzene toxicity as toluene is a competitive inhibitor of benzene metabolism (Andrew et al. 1977). Prior or co-exposure to other chemicals is also likely to modulate the benzene metabolism, suggesting synergistic or antagonistic effect of one or the other chemicals (Medinsky et al. 1994). Benzene, toluene and xylene toxicity mechanism depends on their metabolites (Croute et al. 2002). Benzene-induced toxicity is related to some types of cancer and blood disorders, including bone marrow depression (Wan and Winn 2004; Wetmore et al. 2008). BTX induces genotoxicity and apoptosis in in vitro and in vivo models (Smith 1996; Ross 2000; Snyder 2000; Nakai et al. 2003; Al-Ghamdi et al. 2004; Wan and Winn 2004; Wetmore et al. 2008).

3.2 Benzene

Benzene has a diverse ring and is the smallest and is the most stable aromatic hydrocarbon with no side chain. Benzene is classified as a class I carcinogen that causes DNA damage both in vitro and in vivo (Sul et al. 2005; Weaver et al. 2007; Weaver and Liu 2008). Benzene toxicity is the result of synergistic interaction among metabolites (Smith et al. 1989; Irons et al. 1992; Kolachana et al. 1993;

Chapman et al. 1994), like phenol, hydroquinone and catechol (Medinsky et al. 1996; Snyder 2007). There is significant increase in the chromosomal breaks in K-562 cells, haematopoietic stem cells (HSC), sister chromosomal exchanges and clastogenicity when exposed to benzene and its metabolites (Singh and Winn 2008; Faiola et al. 2004; Erexson et al. 1986; Zhang et al. 2002). Applying comet assay, benzene and its metabolites showed increased comet parameters in the human lymphocytes and HeLa cells (Chen et al. 2008; Galvan et al. 2008). Benzene occupational and environmental hazard is associated with increased risk of leukaemia. Benzene is recognised as a hematotoxin and human carcinogen. (Irons et al. 2013; Lagorio et al. 2013; Li and Yin 2006; Snyder 2012) and is an important pollutant in indoor air (Rappaport et al. 2013; Weisel 2010; Xing et al. 2013). Benzene toxicity is recognised by oxidative stress, which mediates change in DNA methylation (Ahmed et al. 2009; Adami et al. 2006a, b; Ayalogu et al. 2001; Lippmann et al. 2011; Revilla et al. 2007; Brautbar et al. 2006; Saadat and Ansari-Lari 2005; Dogru et al. 2007). 1,4-Benzoquinone (1,4-BQ) is the benzene's most toxic metabolite and is used to evaluate the benzene-induced toxicity (Das et al. 2010; Hu et al. 2014; Stokes and Winn 2014; Tian et al. 2012; Yang and Zhou 2010). Aberrant DNA methylation helps in the early diagnosis of the disease (Deng and Liu 2010). Previous reports documented benzene metabolism-induced reactive oxygen species (ROS) (Badham and Winn 2010), which increases free radicals and attacks DNA molecule, resulting in DNA strand break and oxidative damage (Atkinson 2009; Barreto et al. 2009).

3.3 Xylene, Toluene and Styrene

These are aromatic hydrocarbons with diverse side chain. These including benzene are the most dangerous component of petrol (Perigo and Prado 2005). Risk of acute or chronic toxicity is associated with production distribution and use of petrol (Bruckner and Warren 2001). Their exposure leads to hepatotoxicity and nephrotoxicity (Benson et al. 2011).

3.4 Cytotoxicity Induced by Individual of Benzene, Toluene and Xylene on *Drosophila*

Benzene, toluene and xylene when treated independently on *Drosophila melanogaster* showed that benzene is more toxic than toluene and xylene (Singh et al. 2009, 2011) (Fig. 3.1). As evident by β-*galactosidase* activity after 24 h, test chemicals showed concentration and time-dependent significant induction of the hsp's. Hsp70 is more in concentration than hsp83, which is greater than or equal to hsp26. Test chemicals affected the emergence of the adult flies and significant decrease in the number of flies. This change may be due to the genetic and environmental factors (Gayathri and Krishnamurthy 1981). Benzene affects the reproductive competency of *Drosophila* in most of the cases, which may be due to the damage caused

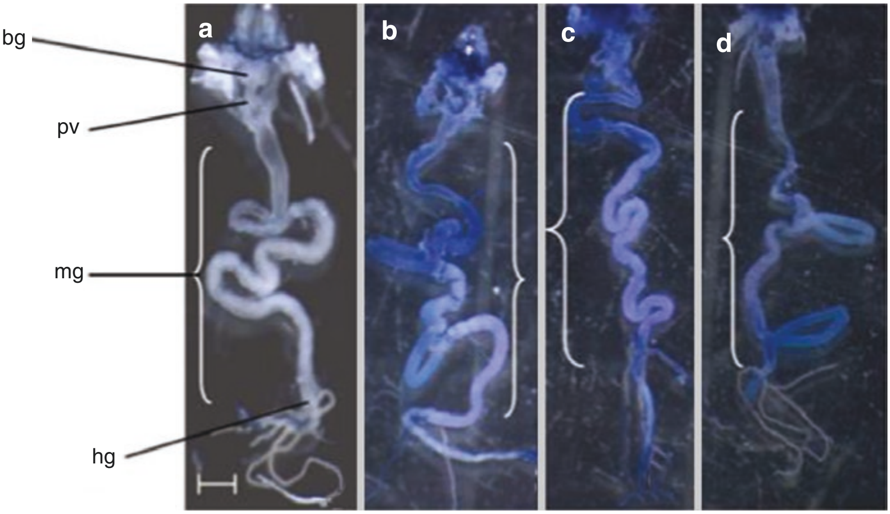


Fig. 3.1 Trypan blue staining in the internal tissues isolated from third instar larvae of *D. melanogaster* (Oregon R+) control (**a**) and in third instar larvae exposed to 100.0 mM benzene (**b**), toluene (**c**) and xylene (**d**) after 48 h. *bg* brain ganglia, *pv* proventriculus, *mg* midgut, *hg* hind gut. *Bar* represents 100 µm (taken from Singh et al. 2011)

by the excessive production of ROS. Flies exposed to higher concentration showed sluggish movement after 48 h. The medium concentration of the test chemicals delayed emergence of the flies by 3 days, medium by 2 days and less concentration delayed emergence by 1 day. Benzene exposure showed the maximum effect on the mean daily egg laying, and there is no significant difference between toluene and xylene exposure. Reproductive performance decreases with the increase in test chemical concentration. At very low concentration, benzene and xylene affected the reproductive performance as compared to toluene. No significant difference was evident between benzene and xylene exposure.

Larvae exposed to the highest concentration of benzene showed regression in β-*galactosidase* activity after 48 h as compared to maximum activity after 24 h. There is no regression in β-*galactosidase* activity after 48 h in xylene and toluene. There is significant difference in β-*galactosidase* activity in benzene and xylene and benzene- and toluene-exposed groups and no significant difference between toluene- and xylene-treated groups. Benzene being the highest inducer for stress genes in larvae compared to xylene and toluene. The highest concentration of benzene exposure to larvae shows maximum increase in the ROS generation as compared to xylene and toluene and no significant difference between the xylene- and toluene-treated groups. The highest concentration triggered significant *catalase* (CAT) activity in larvae after 4 h of exposure to benzene as compared to 6 h when exposed to xylene and toluene. Larvae exposed to low concentration of test chemicals showed a significant depletion in the Glutathione content and total protein content and increase in malondialdehyde content after 12 h in benzene and 24 h in both

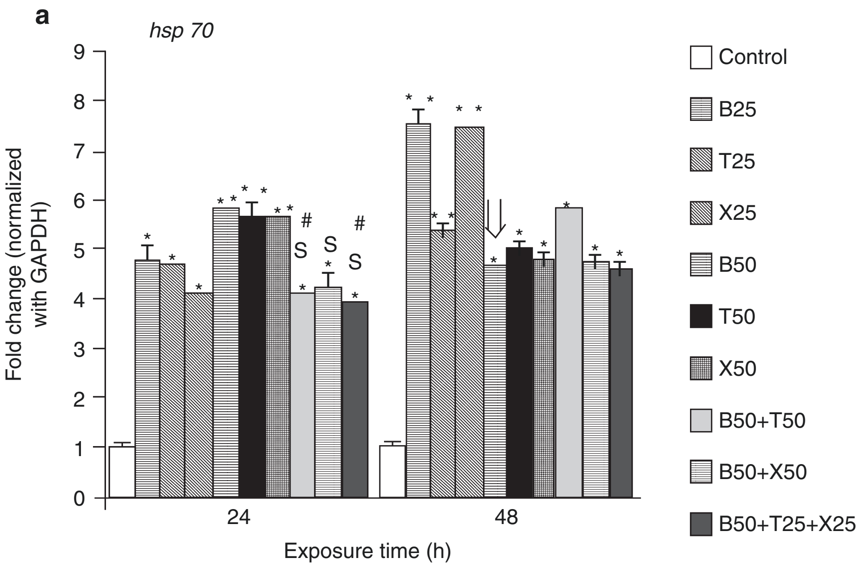
xylene and toluene. A maximum depletion in GSH content was observed after 48 h at the highest dietary concentration of the benzene, toluene and xylene. Larvae exposed to the highest concentration of test chemicals exhibited a significantly enhanced enzyme activity after 6 h in benzene and 12 h in both xylene and toluene. BTX induced cellular and organismal toxicity in *Drosophila* by the expression of stress, oxidative stress and organismal assays.

3.5 Co-exposure of Benzene, Toluene and Xylene (BTX) Mixture on *Drosophila*

Flies were exposed to either individual test chemical or in a mixture of two or three chemicals, i.e. benzene-toluene, benzene-xylene or benzene-toluene and xylene (Singh et al. 2010. The magnitude of toxicity of a benzene-toluene (BT) or benzene-xylene (BX) or benzene-toluene-xylene mixture (BTX) was statistically significantly lower in flies than in the individual chemical cases. When BT is induced, it's seen it has protective effect on mice as toluene is a competitive inhibitor of benzene metabolism. Benzene metabolism is modulated by co-exposure or prior exposure to other chemicals (Medinsky et al. 1994). Combination of toluene and xylene exposure results in additive effect (Chen et al. 1994). There is a reduction in benzene-induced cellular and genetic toxicity when exposed to BT or BX mixture (Gad-El-Karim et al. 1984; Plappert et al. 1994).

We did not observe any mortality in any of the mixture concentration throughout the exposure duration (Singh et al. 2010). In mixture-treated groups, they observed the delay in the emergence of the flies by 1 day. BT, BX and BTX mixture-treated groups also show significantly lower β-*galactosidase* activity. Levels of hsp70 in mixture-treated groups also increased significantly after 48 h as compared to that after 24 h (Fig. 3.2). ROS generation and CAT activity were significantly lower in mixture-treated group as compared to the individual tested groups. They showed that benzene, toluene and xylene either individually or in mixture are toxic to the exposed organism, but the mixture is less toxic than the individual chemicals. The highest concentrations of all the components of mixtures cause 100% larval lethality after 2 h indicating a very severe additive effect of the mixture. This indicates that after threshold limit, these may work synergistically or additively with benzene. The exposed organism was rescued means organismal toxicity was less severe when larvae were exposed to BT, BX or BTX mixture. Mixed chemicals affect the hsp70 expression only after 48 h as compared to individual chemical exposure which is after 24 h. This indicates lesser generation of damaging signals in cells when treated by mixed chemicals. BTX, BT and BX mixture decreased the activities of CAT, MDA and PC content and lesser depletion of GSH content in the exposed organism.

Exposure of organisms to the pollutants, which includes monocyclic aromatic hydrocarbons, shows genotoxicity and apoptosis. Genotoxicity is defined as a destructive effect on a cells genetic material that is DNA and RNA, affecting its integrity. Apoptosis is the death of cells which occurs as a normal and controlled



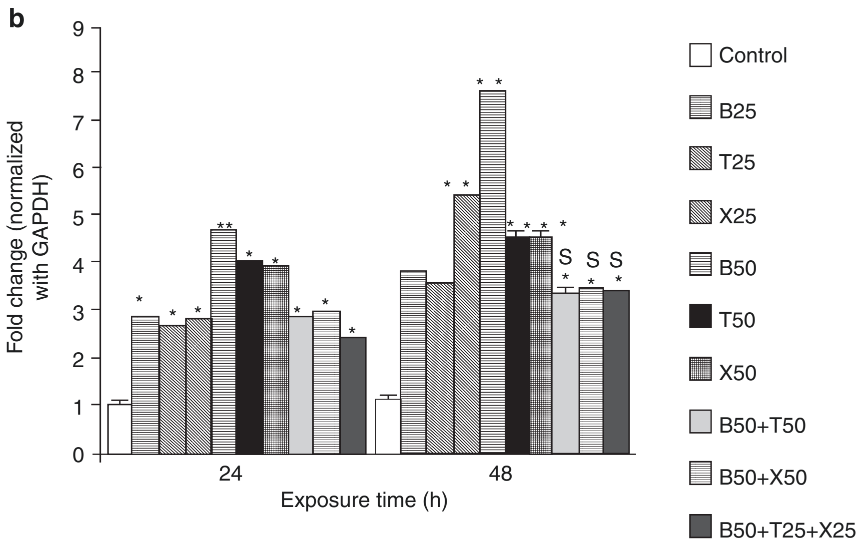


Fig. 3.2 Quantitative real-time PCR (qRT-PCR) analysis of hsp70 (**a**), *hsp83* (**b**), h*sp60* (**c**) and *hsp26* (**d**) mRNA in control, individual and mixtures exposed third instar larvae of *D. melanogaster* (Oregon R+) for 24 and 48 h (taken from Singh et al. 2010)

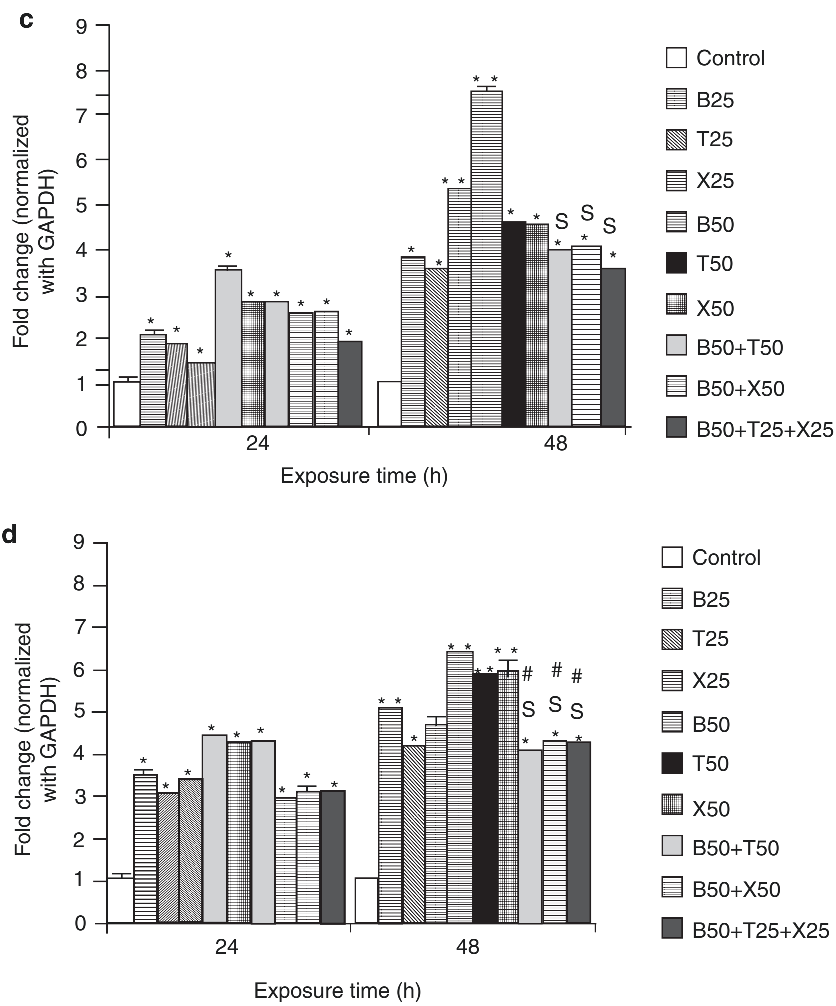


Fig. 3.2 (continued)

part of an organism's development and growth. Comet assay parameters show damage only after 24 h in benzene and 48 h in toluene and xylene. There is significantly increase in the AV-positive cells when exposed to low concentration of benzene after 24 h and after 48 h in xylene and toluene. At the highest concentration, this increase is after 12 h and 24 h, respectively. For *caspase* activity change in mitochondrial membrane potential is required (Hay and Guo 2006). Larvae when exposed to low concentration of benzene exhibit significant change in membrane potential after 24 h and XT showed after 48 h. BTX showed this effect at high concentration after 24 h.

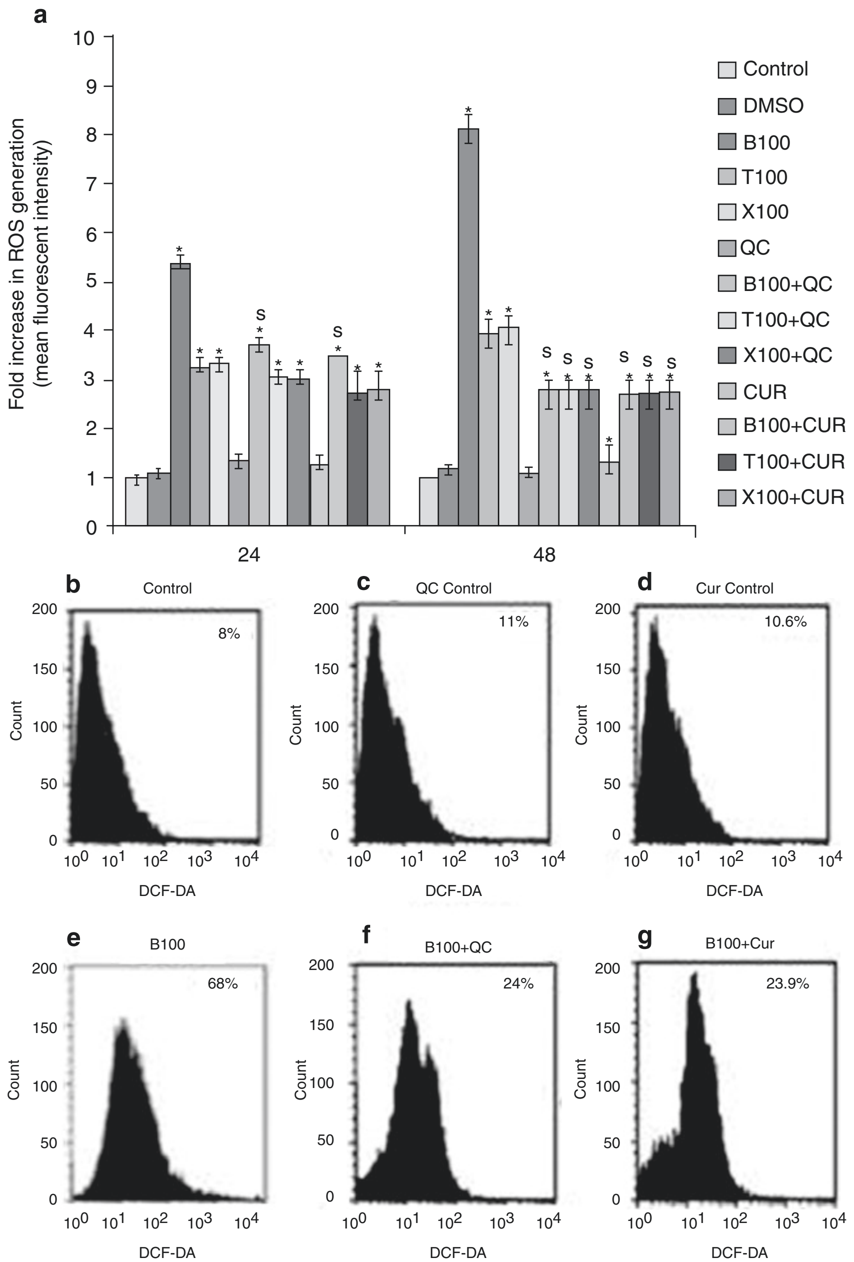


Fig. 3.3 ROS generation in D. *melanogaster* (Oregon R+) in control, DMSO and benzene, toluene or xylene alone or in combinations with QC or CUR treatments for 24 and 48 h. Histogram (**a**) depicts ROS generation in test chemical exposed organisms, and flow cytometric panels show the ROS generation in (**b**) control, (**c**) QC control, (**d**) CUR control, (**e**) B100, (**f**) B100 + QC and (**g**) B100 + CUR exposed organisms after 48 h. Data represent mean ± SD of three identical experiments made in triplicates, and significance is ascribed as $^*P < 0.01$ vs. control; $^{\$}P < 0.01$, reduction vs. individual chemical (B100 or T100 or X100) (taken from Singh et al. 2011)

Larvae when exposed to curcumin and quercetin mixed with chemical mixtures show significant diminution in the GST activity and reduction in ROS generation, SOD, CAT activity and MDA content after 48 h. There is also significant reduction in DNA damage and DNA migration after 24 and 48 h when exposed to QC and CUR (Fig. 3.3).

3.6 *Drosophila*: A Tool for Future Perspective

Drosophila melanogaster is an insect which belongs to phylum Arthropoda, class Insect and order Diptera. *Drosophila* is accepted as an animal model because of its defined genetics and molecular biology. This genetically defined organism is a front-runner. It is widely used in studying genetics, life history evolution and developmental biology. Recently fruit fly is used in modelling human diseases. Its genome can be easily manipulated for studying a particular gene of interest under a defined condition. Recent studies showed that this can be used for toxicological studies of various environmental pollutants. Fly and higher mammals have a similar dose-response relationship with four monofunctional alkylating agents (Siddique et al. 2005). *Drosophila* have small size of 3 mm in length, and its larvae increase around 1000-fold in weight in 5–6 days. *Drosophila* has many genetic mutations including many different eye colours that are very useful for geneticists. Female lays 30–50 eggs/day throughout her lifetime. Genome sequence of *Drosophila* was completed in 2000. Its genome has 168,736,537 base pairs and ~14,000 protein-coding genes. Approximately 75% of the genes that are causing diseases in humans are also found in fruit fly. It has short and simple reproductive cycle. This means several generations can be studied in a matter of month. The only care they need is that their food has to be changed regularly. Thus they are inexpensive and easy to maintain in the laboratory.

References

Adami G, Larese F, Venier M, Barbieri P, LoCoco F, Reisenhofer E (2006a) Penetration of benzene, toluene and xylenes contained in gasoline's through human abdominal skin in vitro. Toxicol In Vitro 20:1321e30

Adami G, Larese F, Venier M, Barbieri P, Lo Coco F, Reisenhofer E (2006b) Penetration of benzene, toluene and xylenes contained in gasolines through human abdominal skin in vitro. Toxicol In Vitro 20:1321–1330

Ahmed HH, Metwally FM, Rashad HM (2009) Toxicity of solvents exposure on the neuroendocrine system in rats: role of amino acids supplementation. Toxic Solvents Rep Opin 1:66e83

Al-Ghamdi SS, Raftery MJ, Yaqoob MM (2004) Toluene and p-xylene induced LLCPK1 apoptosis. Drug Chem Toxicol 27:425–432

Andrew LS, Lee EW, Witmer CM, Kocsis JJ, Snyder R (1977) Effects of toluene on the metabolism, disposition and hemopoietic toxicity of [3H] benzene. Biochem Pharmacol 26:293–300

Atkinson TJ (2009) A review of the role of benzene metabolites and mechanisms in malignant transformation: summative evidence for a lack of research in nonmyelogenous cancer types. Int J Hyg Environ Health 212:1–10

Ayalogu OE, Igboh NM, Dede EB (2001) Biochemical changes in the serum and liver of albino rats exposed to petroleum samples (gasoline, kerosene, and crude petroleum). J Appl Sci Environ Manage 5:97e100

Badham HJ, Winn LM (2010) In utero exposure to benzene disrupts fetal hematopoietic progenitor cell growth via reactive oxygen species. Toxicol Sci 113:207–215

Barreto G, Madureira D, Capani F, Aon-Bertolino L, Saraceno E, Alvarez-Giraldez LD (2009) The role of catechols and free radicals in benzene toxicity: an oxidative DNA damage pathway. Environ Mol Mutagen 50:771–780

Benson JM, Gigliotti AP, March TH, Barr EB, Tibbetts BM, Skipper BJ, Clark CR, Twerdok L (2011) Chronic carcinogenicity of gasoline vapour condensate (GVC) and GVC containing methyl tertiary-butyl ether in f344 rats. J Toxicol Environ Health A 74:638e57

Brautbar N, Wu MP, Gabel E, Regev L (2006) Occupational kidney cancer exposure to industrial solvents. Ann N Y Acad Sci 1076:753e64

Bruckner JV, Warren DA (2001) Toxic effects of solvents and vapors. In: Klaassen CD (ed) Casarette and Doulls toxicology the basic science of poisons, 6th edn. McGraw-Hill Medical, New York, p 869e944

Chang FK, Chen ML, Cheng SF, Shih TS, Mao IF (2007) Dermal absorption of solvents as a major source of exposure among shipyard spray painters. J Occup Environ Med 49:430–436

Chapman DE, Namkung MJ, Juchau MR (1994) Benzene and benzene metabolites as embryotoxic agents: effects on cultured rat embryos. Toxicol Appl Pharmacol 128:129–137

Chen Z, Liu SJ, Cai SX, Yao YM, Yin H, Ukai H, Uchida Y, Nakatsuka H, Watanabe T, Ikeda M (1994) Exposure of workers to a mixture of toluene and xylenes. II. Effects. Occup Environ Med 51:47–59

Chen Y, McMillan-Ward E, Kong J, Israels SJ, Gibson SB (2008) Oxidative stress induces autophagic cell death independent of apoptosis in transformed and cancer cells. Cell Death Differ 15:171–182

Chou CC, Riviere JE, Monteiro-Riviere NA (2003) The cytotoxicity of jet fuel aromatic hydrocarbons and dose-related interleukin-8 release from human epidermal keratinocytes. Arch Toxicol 77:384–391

Croute F, Poinsot J, Gaubin Y, Beau B, Simon V, Murat JC, Soleilhavoup JP (2002) Volatile organic compounds cytotoxicity and expression of HSP72, HSP90 and GRP78 stress proteins in cultured human cells. Biochim Biophys Acta 1591:147–155

Das S, Chakrabarty D, Choudhury GC (2010) 1,4-benzoquinone (PBQ) induced toxicity in lung epithelial cells is mediated by the disruption of the microtubule network and activation of caspase-3. Chem Res Toxicol 23:1054–1066

Deng Z, Liu YD (2010) Epigenetic alterations as cancer diagnostic, prognostic, and predictive biomarkers. Adv Genet 71:125–176

Derwent RG, Davies TJ, Delaney M, Dollard GJ, Field RA, Dumitrean P, Nason PD, Jones BMR, Pepler SA (2000) Analysis and interpretation of the continuous hourly monitoring data for 26 C2–C8 hydrocarbons at 12 United Kingdom sites during 1996. Atmos Environ 34:297–312

Dogru O, Celkan T, Demir T (2007) Hematological and biochemical changes in volatile substance abusing street children in Istanbul. Turk J Hematol 24:52e6

Dosemeci M, Li GL, Hayes RB, Yin SN, Linet M, Chow WH, Wang YZ, Jiang ZL, Dai TR, Zhang WU (1994) Cohort study among workers exposed to benzene in China: II. Exposure assessment. Am J Ind Med 26:401–411

Erexson GL, Wilmer JL, Steinhagen WH, Kligerman AD (1986) Induction of cytogenetic damage in rodents after short-term inhalation of benzene. Environ Mutagen 8:29–40

Faiola B, Fuller ES, Wong VA, Pluta L, Abernethy DJ, Rose J, Recio L (2004) Exposure of hematopoietic stem cells to benzene or 1,4-benzoquinone induces gender-specific gene expression. Stem Cells 22:750–758

Gad-El-Karim MM, Harper BL, Legator MS (1984) Modifications in the myeloclastogenic effect of benzene in mice with toluene, phenobarbital, 3- methylcholanthrene, Aroclor 1254, SKF-525A. Mutat Res 135:225–243

Gallegos P, Lutz J, Markwiese J, Ryti R, Mirenda R (2007) Wildlife ecological screening levels for inhalation of volatile organic chemicals. Environ Toxicol Chem 26:1299–1303
Galvan N, Lim S, Zmugg S, Smith MT, Zhang L (2008) Depletion of WRN enhances DNA damage in HeLa cells exposed to the benzene metabolite, hydroquinone. Mutat Res 649:54–61
Gayathri MV, Krishnamurthy NB (1981) Studies on the toxicity of mercurial fungicide Agallol3 in Drosophila melanogaster. Environ Res 24:89–95
Greer JE (1984) Adolescent abuse of typewriter correction fluid. South Med J 77:297–298
Hay BA, Guo M (2006) Caspase-dependent cell death in Drosophila. Annu Rev Cell Dev Biol 22:623–650
Hu J, Ma H, Zhang W, Yu Z, Fu J (2014) Effects of benzene and its metabolites on global DNA methylation in human normal hepatic L02 cells. Environ Toxicol 29:108–116
Irons RD, Stillman WS, Colagiovanni DB, Henry VA (1992) Synergistic action of benzene metabolite hydroquinone on myelopoietic stimulating activity of granulocyte/macrophage colony-stimulating in vitro. Proc Natl Acad Sci U S A 89:3691–3695
Irons RD, Chen Y, Wang X, Ryder J, Kerzic PJ (2013) Acute myeloid leukemia following exposure to benzene more closely resembles de novo than therapy related-disease. Genes Chromosomes Cancer 52:887–894
Kim KH, Kim MY (2002) The distributions of BTEX compounds in the ambient atmosphere of the Nan-Ji-Do abandoned landfill site in Seoul. Atmos Environ 36:2433–2446
Kolachana P, Subrahmanyam VV, Meyer K, Zhang L, Smith M (1993) Benzene and its phenolic metabolites produce oxidative DNA damage in HL-60 cells in vitro and in the bone marrow in vivo. Cancer Res 53:1023–1026
Kozel N, Sloboda Z, De La Rosa M (eds) (1995) Epidemiology of inhalant abuse: an international perspective. US Department of Health and Human Services, Washington, DC. NIDA Res. Mon. 14
Kuang S, Liang W (2005) Clinical analysis of 43 cases of chronic benzene poisoning. Chem Biol Interact 30:129–135
Lagorio S, Ferrante D, Ranucci A, Negri S, Sacco P, Rondelli R, Cannizzaro S, Torregrossa MV, Cocco P, Forastiere F, Miligi L, Bisanti L, Magnani C (2013) Exposure to benzene and childhood leukaemia: a pilot case-control study. BMJ Open 3:e002275
Lee SC, Chiu MY, Ho KF, Zou SC, Wang X (2002) Volatile organic compounds (VOCs) in urban atmosphere of Hong Kong. Chemosphere 48:375–382
Li G, Yin S (2006) Progress of epidemiological and molecular epidemiological studies on benzene in China. Ann N Y Acad Sci 1076:800–809
Lippmann SJ, Richardson DB, Chen JCM (2011) Elevated serum liver enzymes and fatty liver changes associated with long driving among taxi drivers. Am J Ind Med 54:618e27
Medinsky MA, Schlosser PM, Bond JA (1994) Critical issues in benzene toxicity and metabolism: the effect of interactions with other organic chemicals on risk assessment. Environ Health Perspect 102:119–124
Medinsky MA, Kenyon EM, Seaton MJ, Schlosser PM (1996) Mechanistic considerations in benzene physiological model development. Environ Health Perspect 104:1399–1404
Nakai N, Murata M, Nagahama M, Hirase T, Tanaka M, Fujikawa T, Nakao N, Nakashima K, Kawanishi S (2003) Oxidative DNA damage induced by toluene is involved in its male reproductive toxicity. Free Radic Res 37:69–76
Perigo JF, Prado C (2005) Evolution of occupational exposure to environmental levels of aromatic hydrocarbons in service stations. Ann Occup Hyg 49(233e):40
Plappert U, Barthel E, Seidel HJ (1994) Reduction of benzene toxicity by toluene. Environ Mol Mutagen 24:283–292
Rappaport SM, Kim S, Thomas R, Johnson BA, Bois FY, Kupper LL (2013) Low-dose metabolism of benzene in humans: science and obfuscation. Carcinogenesis 34:2–9
Revilla AS, Pestana CR, Pardo-Andreu GL, Santos AC, Uyemura SA, Gonzales ME (2007) Potential toxicity of toluene and xylene evoked by mitochondrial uncoupling. Toxicol In Vitro 21:782e8
Ross D (2000) The role of metabolism and specific metabolites in benzene-induced toxicity: evidence and issues. J Toxicol Environ Health A 61:357–372

Saadat M, Ansari-Lari M (2005) Alterations of liver function test indices of filling station workers with respect of genetic polymorphisms of GSTM1 and GSTT1. Cancer Lett 227:163e7
Siddique HR, Kar Chowdhuri D, Saxena DK, Dhawan A (2005) Validation of Drosophila melanogaster as an in vivo model for genotoxicity assessment using modified alkaline Comet assay. Mutagenesis 4:285–290
Singh R, Winn LM (2008) The effects of 1,4-benzoquinone on c-Myb and topoisomerase II in K-562 cells. Mutat Res 645:33–38
Singh MP, Reddy MM, Mathur N, Saxena DK, Chowdhuri DK (2009) Induction of hsp70, hsp60, hsp83 and hsp26 and oxidative stress markers in benzene, toluene and xylene exposed Drosophila melanogaster: role of ROS generation. Toxicol Appl Pharmacol 235(2):226–243
Singh MP, Ram KR, Mishra M, Shrivastava M, Saxena DK, Chowdhuri DK (2010) Effects of co-exposure of benzene, toluene and xylene to Drosophila melanogaster: alteration in hsp70, hsp60, hsp83, hsp26, ROS generation and oxidative stress markers. Chemosphere 79(5):577–587
Singh MP, Mishra M, Sharma A, Shukla AK, Mudiam MK, Patel DK, Ram KR, Chowdhuri DK (2011) Genotoxicity and apoptosis in Drosophila melanogaster exposed to benzene, toluene and xylene: attenuation by quercetin and curcumin. Toxicol Appl Pharmacol 253(1):14–30
Smith MT (1996) Mechanistic studies of benzene toxicity—implications for risk assessment. Adv Exp Med Biol 387:259–266
Smith MT, Yager JW, Steinmetz KL, Eastmond DA (1989) Peroxidase-dependent metabolism of benzene's phenolic metabolites and its potential role in benzene toxicity and carcinogenicity. Environ Health Perspect 82:23–29
Snyder R (2000) Overview of the toxicology of benzene. J Toxicol Environ Health A 61:339–346
Snyder R (2007) Benzene's toxicity: a consolidated short review of human and animal studies by HA Khan. Hum Exp Toxicol 26:687–696
Snyder R (2012) Leukemia and benzene. Int J Environ Res Public Health 9:2875–2893
Spiller HA, Krenzelok EP (1997) Epidemiology of inhalant abuse reported to two regional poison centers. J Toxicol Clin Toxicol 35:167–173
Srivastava A, Devotta S (2007) Indoor air quality of public places in Mumbai, India in terms of volatile organic compounds. Environ Monit Assess 133:127–138
Stokes SE, Winn LM (2014) NF-kappaB signaling is increased in hd3 cells following exposure to 1,4-benzoquinone: role of reactive oxygen species and p38-MAPK. Toxicol Sci 137:303–310
Sul D, Lee E, Lee MY, Oh E, Im H, Lee J, Jung WW, Won N, Kang HS, Kim EM, Kang SK (2005) DNA damage in lymphocytes of benzene exposed workers correlates with trans, trans-muconic acids and breath benzene levels. Mutat Res 582:61–70
Tian JF, Peng CH, Yu XY, Yang XJ, Yan HT (2012) Expression and methylation analysis of p15 and p16 in mouse bone marrow cells exposed to 1,4-benzoquinone. Hum Exp Toxicol 31:718–725
US EPA 1996 Priority pollutants, code of federal regulations. Title 40; U.S. Environmental Protection Agency. U.S. Government Printing Office, Washington, DC. Part 423, App. A (chapter 1)
Wan J, Winn LM (2004) The effects of benzene and the metabolites phenol and catechol on c-Myb and Pim-1 signaling in HD3 cells. Toxicol Appl Pharmacol 201:194–201
Wang L, Zhou Y, Liang Y, Wong O, Armstrong T, Schnatter AR, Wu Q, Fang J, Ye X, Fu H, Irons RD (2006) Benzene exposure in the shoemaking industry in China, a literature survey, 1978–2004. Regul Toxicol Pharmacol 46:149–156
Weaver CV, Liu SP (2008) Differentially expressed pro- and anti-apoptogenic genes in response to benzene exposure: immunohistochemical localization of p53, Bag, Bad, Bax, Bcl-2, and Bcl-w in lung epithelia. Exp Toxicol Pathol 59:265–272
Weaver CV, Liu SP, Lu JF, Lin BS (2007) The effects of benzene exposure on apoptosisin epithelial lung cells: localization by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) and the immunocytochemical localization of apoptosis-related gene products. Cell Biol Toxicol 23:201–220
Weisel CP (2010) Benzene exposure: an overview of monitoring methods and their findings. Chem Biol Interact 184:58–66

Wetmore BA, Struve MF, Gao P, Sharma S, Allison N, Roberts KC, Letinski DJ, Nicolich MJ, Bird MG, Dorman DC (2008) Genotoxicity of intermittent co-exposure to benzene and toluene in male CD-1 mice. Chem Biol Interact 173:166–178

Wong O, Raabe GK (1989) Critical review of cancer epidemiology in petroleum industry employees, with a quantitative meta-analysis by cancer site. Am J Ind Med 15:283–310

Xing Q, Chen G, Li L, Zhang M, Zheng Z, Zou L, Hou QF, Wang X, Liu XG (2013) Microsomal epoxide hydrolase (EPHX1) polymorphisms are associated with aberrant promoter methylation of ERCC3 and hematotoxicity in benzene-exposed workers. Environ Mol Mutagen 54:397–405

Yang F, Zhou JH (2010) Cytotoxicity and DNA damage induced by 1,4-benzoquinone in v79 Chinese hamster lung cells. J Toxicol Environ Health A 73:483–489

Zhang L, Eastmond DA, Smith MT (2002) The nature of chromosomal aberrations detected in humans exposed to benzene. Crit Rev Toxicol 32:1–42

Tracing of Evolution in Silkworm, *Bombyx mori* L., on the Basis of Molecular Studies

4

E. Muniraju and Rajendra Mundkur

Abstract

Pure Mysore and Nistari are known oldest races of India. Both are multivoltine races which produce colored cocoons. The origin of these races is obscure. Pure Mysore was supposed to have originated from the race that Mysore King Tippu Sultan brought from China in 1875 and established in Karnataka State. Nistari is the well-known race also believed to have brought from China and established in West Bengal area. However, the wild sericigenous species of *Bombyx*, *Theophila*, and *Ocinara* are naturally distributed in the Himalayan ranges of Indo-China range and also in the Andaman Islands in India, besides Jawa, Sumatra, Borneo, and Malay Peninsula (Barlow, An introduction to the moths of South East Asia, 1982). Apart from these, there are wild relatives of silkworm, *B. mandarina*, which have been collected from Kedarnath. It is believed that the silkworm, *Bombyx mori* L., has evolved from *B. mandarina* in China and spread across the globe. There are many theories about the pattern of silkworm evolution and spread. In this article, the theories are discussed on the molecular basis.

4.1 Introduction

Pure Mysore and Nistari are known oldest races of India. Both are Multivoltine races which produce colored cocoons. The origin of these races is obscure. Pure Mysore was supposed to have originated from the race that Mysore King Tippu Sultan brought from China in 1875 and established in Karnataka State. Nistari is the well-known race also believed to have brought from China and established in

E. Muniraju (✉) • R. Mundkur
Karnataka State Sericulture Research and Development Institute,
Thalaghattapura, Bangalore 560 109, India
e-mail: emuniraju@yahoo.com

D. Kumar, C. Gong (eds.), *Trends in Insect Molecular Biology and Biotechnology*, https://doi.org/10.1007/978-3-319-61343-7_4



Fig. 4.1 Geographical location of Kedarnath where Ancestor of Silkworm, *Bombyx mandarina* is also available in the wild

West Bengal area. However, the wild sericigenous species of *Bombyx, Theophila,* and *Ocinara* are naturally distributed in the Himalayan ranges of Indo-China range and also in Andaman Islands in India, besides Jawa, Sumatra, Borneo, and Malaya Peninsular (Barlow 1982). In addition to these species, wild relatives of silkworm, *B. mandarina*, has been collected from Kedarnath (Fig. 4.1). It is believed that the Silkworm, *Bombyx mori* L. has evolved from *B. mandarina* in China and spread across the globe. There are many theories about the pattern of silkworm evolution and spread. In this article, the theories are discussed on the molecular basis.

4.2 Voltinism

Voltinism is one of the important factors for discussing about the evolution and adaptation of the silkworm. The silkworm, *Bombyx mori* is classified based on the geographical regions, as Temperate and Tropical races. They are classified on the basis of voltinism as Univoltines, Bivoltines, and Multivoltines (or Polyvoltines); depending upon the number of generations, they undergo in a year. Generally, tropical silkworms are polyvoltines and the polyvoltine eggs do not undergo hibernation, which means that embryos develop continuously and hatch in about 10–11 days after egg laying. In univoltines and bivoltines, the embryos "pause" development and enter hibernation at blastoderm stage which is about 20–22 h after oviposition. Univoltines are temperate silkworm races, show *facultative* type of diapause wherein the eggs exhibit diapause phenomenon irrespective of environmental factors. Bivoltines are also temperate races which show *obligatory* type of diapause wherein diapause phenomenon is modified by environmental factors (Fig. 4.2).



Fig. 4.2 Map showing region-wise voltinism in silkworm

Dominance of voltinism is recognized in the order of Univoltines > Bivoltine > Multivoltine. The multivoltine races always lay nonhibernating eggs. The breeds between multi- and bivoltine could lay some non-hibernating eggs under high incubation temperatures. The bivoltine strains lay non-hibernating eggs under low and hibernating eggs under high temperature of egg incubation. The breeds between bi- and univoltine could lay both non- and hibernating eggs, even under low incubation temperatures. The typical univoltine races always lay hibernating eggs (Morohoshi 2000).

Andrewartha (1952) opines that diapause is a convergent trait that has evolved independently several times in the course of evolution. Toyama (1906a, 1913) described diapause as a maternally inherited biological event under the control of sex-linked genes. Sonobe and Odake (1986) have proposed two theories related to the embryonic diapause in silkworm: (1) diapause is the phenomenon predetermined by the diapause factor during embryogenesis, and (2) diapause is the process determined by the genetic factor during embryogenesis. Mundkur et al. (2010a, b) have proposed a new theory related to diapause specially taking paternal influence into account. "diapause is the phenomenon determined by environment, maternal genes and paternal genes synergistically or exclusively" (Figs. 4.3, 4.4, and 4.5).

Understanding these theories of silkworm diversification is important to silkworm breeders. Just like dominance of voltinism, the productivity and quality is also in the order of Univoltines > Bivoltine > Multivoltine. Tropical multivoltines are inferior in productivity. Indian breeders look towards sub-tropical bivoltines to improve the productive and qualitative traits. If the bivoltines of subtropics are introduced to Indian tropical conditions, they fail to behave as in their original environment. Over the period of time they lose their productive qualities and turn themselves into polyvoltines (Table 4.1).



Fig. 4.3 Bivoltine to multivoltine theory of evolution (Gamo and Ohtsuka 1980)



Fig. 4.4 Multivoltine to Bivoltine theory of evolution (Yoshitake 1968)

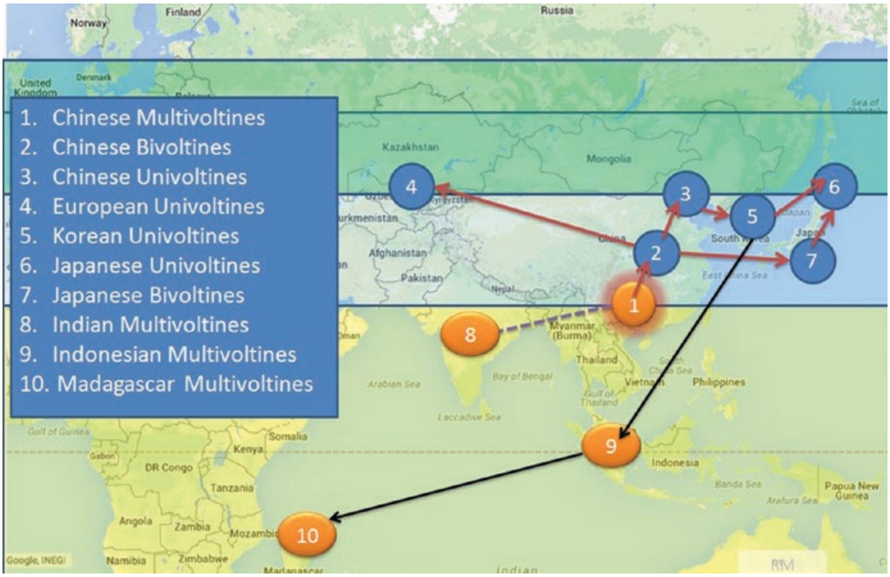


Fig. 4.5 KSSRDI model of silkworm diversification

Table 4.1 Order of merit of four important parameters in silkworm

Voltinism	Univoltine > Bivoltine > Multivoltine
Productivity	Univoltine > Bivoltine > Multivoltine
Quality of raw silk	Univoltine > Bivoltine > Multivoltine
Resistance	Multivoltine > Bivoltine > Univoltine

4.3 Correlation Between Characters

In silkworms, it is observed that one character is influenced by another character generally contrasting one. We can measure their association by correlation studies. Correlations may be positive (i.e., when the quality and quantity of one character go up, those of other character also go up) or negative (i.e., when the quality and quantity of one character go up, those of other character come down). Two major causes of correlation between characters are genetic and environmental factors which result in correlation between the genotypic values of the two characters and the correlation between the environmental deviations (HoZoo 1997). The primary cause of genetic correlation is pleiotropic gene action. Pleiotropy refers to the gene's attribute which affects more than one character, so that if one gene is segregating, it causes simultaneous variation in the character it affects. However, linkage is also a cause of genetic correlation, especially in populations derived from crosses between varieties with divergent genetic backgrounds. Such actions of a gene or interactions gene to gene as well as genotype to environment result in phenotypic, genetic, or environmental

correlations between the characters of economic interest. The extent of correlation usually varies among silkworm breeds and between male and female due to differences in crossover rates which are higher in the male than in the female. For all polygenic traits, the guiding principle for any silkworm breeding scheme is information on correlation and linkage in the female, along with prior knowledge about heritability. Before beginning actual breeding experiments, it is important to know how improvement of one character will cause simultaneous change, either in a positive or negative direction, in other characters. In that aspect the ignoring of mutual genetic link between leading productive traits led to decrease in the genetic progress. Correlation between cocoon weight and pupal weight was high and positive (+0.994), between cocoon weight and shell weight was also positive but lower (+0.614), as well between pupal weight and cocoon shell weight (+0.527). Considerable negative correlation was found between pupal weight and shell ratio (−0.827) (Singh et al. 1992a, b). Fecundity correlates positively with pupal weight but negative with productivity, shell ratio, and strength of fiber. The shell ratio is a better trait for assessing the quality of cocoons for reeling because higher shell ratio led to higher silk yield, due to the proved positive correlation between shell ratio and quality of cocoons.

The phenotypic correlation not always corresponded to the genetic nature and accurately reflected the degree and character of inherited link between productive traits in *Bombyx mori* L. For example, this is the indicated negative phenotypic correlation between traits shell ratio and total egg weight in a batch. In that case negative phenotypic correlation informed that carrying the selection on shell ratio in positive direction will affect negatively on weight of eggs in batches. In the same time however positive genetic correlations show possibilities for differentiation of lines that combine as high shell ratio, as relatively high reproductive ability of silkworm (Grekov and Petkov 1990). The fulcrum relationship of resistance and productivity is of great concern to the breeders. Available data indirectly indicate that they are linked characters, though we don't have the direct genetic proof of it. Various investigators have correlated silkworm characters. Table 4.2 summarizes the inference. Nagaraju (1998) has tabulated (Table 4.3) the correlation of various characters in silkworm based on 15 years of breeding data. It is generalized that, when the productivity goes up, the survivability comes down (Fig. 4.6), but neither Table 4.2 nor Table 4.3 substantiates that concept.

4.4 Survival of the Fit

Fitness involves the ability of organisms (or populations or species) to survive and reproduce in the environment in which they find themselves (Allen Orr 2009). Natural selection is the process by which the best adaptations survive long enough to reproduce. Natural selection is the process by which the traits that are useful for the survival will continue on to the next generation and what does not work will be eliminated. The most important factor influencing the fitness is the environment. Let us take the example of Pure Mysore, with only two parameters, survival and cocoon weight. About 25 years ago, during the 1990s, the average cocoon weight of Pure Mysore cocoon was 1.00 g, and the survival (ERR) was 95%. Today, with improved

Table 4.2 Correlation of characters in silkworm as inferred by various authors

Correlation between	Correlation	References
Fecundity and robustness	Negative	Gowda et al. (1988)
Fecundity and female pupal weight	Positive	Jayaswal et al. (1991)
Cocoon weight and larval weight	Positive	Satenahalli et al. (1990)
Cocoon weight ami shell weight	Positive	Petkov (1981a)
Cocoon weight and filament length	Positive	Petkov (1981c)
Cocoon weight mid pupal weight	Positive	Rajanna and Reddy (1990a)
Cocoon weight and denier	Positive	Satenahalli et al. 1990
Female pupal wt. and larval weight	Positive	Gowda et al. (1989)
Cocoon weight and cocoon silkiness	Positive	Grekov and Petkov (1990)
Shell weight and cocoon weight	Positive	Ozdzenska and Kremky (1987)
Shell weight and pupal weight	Positive	Singh et al. (1992a)
Shell weight and cocoon silkiness	Positive	Long and Petkov (1987)
Shell weight and filament length	Positive	Petkov (1981b)
Shell ratio and cocoon quality	Positive	Singh et al. (1992b)
Shell ratio and pupal weight	Negative	Singh et al. (1992c)
Shell ratio and filament length	Positive	Petkov (1981b)
Filament length and reelability	Negative	Jayaswal et al. (1990)
Yield of cocoons of parent and their hybrids	Positive	Jonaka (1986)
Reelability and tenacity	Positive	Liu and He (1991)
Length and size of filament	Positive	Miyahara (1978)

inputs, the selection could be made up to 1.4 g cocoon weight with the same ERR. However, there is a limit. We cannot match the productivity of temperate bivoltines in temperate conditions (2.0 g cocoon weight with 95% ERR) in Pure Mysore. Let us relocate these two breeds, Pure Mysore to temperate congenial environment and temperate bivoltine to the tropical unfavorable environment. Pure Mysore in temperate conditions will gradually turn into diapausing type and show little higher cocoon weight (1.6 g) than in tropical conditions. It will never match the temperate bivoltine in productivity even if it is placed in the same environment as of temperate bivoltine breed. It looks like it simply does not have the genetic machinery for producing higher cocoon weight. The relocated bivoltine in tropical conditions lose its productivity to retain its survivability (as in case of C-Nichi). The net result is that there is no improvement in survivability, but the productivity suffered. Therefore, the environment plays a major role in deciding the quality and productivity parameters.

4.5 Behavior of Temperate Silkworm Races in Indian Tropical Conditions

In Japan, silkworm eggs hatch after completion of hibernation during spring (about middle of May), in warmer climate with about 20°C temperature. They turn into moths around later half of June and lay eggs. The deposited eggs are nonpigmented pale yellow in color and nonhibernating. They hatch and grow during early July,

Table 4.3 Correlation of 16 characters in silkworm (Nagaraju 1998)

SI. no.	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	−0.150	−0.20	−0.046	0.054	−0.126	−0.124	−0.154	−0.165	−0.155	−0.073	0.160	0.086	0.218	0.022	−0.121
2		0.00	−0.377	−0.433	−0.361	−0.343	−0.079	−0.303	−0.432	−0.477	0.175	0.054	−0.339	−0.799	−0.332
3	*		0.259	−0.249	−0.278	0.001	0.452	0.256	0.066	−0.130	−0.235	0.195	−0.178	−0.260	−0.365
4	–*			0.416	0.121	0.144	0.103	0.341	0.288	0.159	−0.277	0.215	−0.194	0.450	0.097
5	–*				0.871	0.710	−0.011	0.097	0.399	0.638	0.109	−0.319	−0.033	0.752	0.857
6	–*			***		0.852	0.084	0.184	0.472	0.771	0.140	−0.368	0.104	0.705	0.975
7	–			***	***		0.586	0.544	0.665	0.918	0.019	−0.231	0.129	0.760	0.715
8		**				***		0.781	0.547	0.554	−0.198	0.106	0.054	0.350	−0.135
9	–		–			**	***		0.672	0.691	−0.257	0.013	0.068	0.561	0.019
10	–*			**	**	***	**	***		0.750	−0.678	−0.122	0.240	0.671	0.349
11	–**			***	***	***	***	***	***		−0.028	−0.199	0.144	0.839	0.642
12									***			0.421	−0.248	−0.107	0.180
13				–	–*						–*		0.005	−0.150	−0.395
14	–													0.161	0.083
15	***		**	***	***	***	*	***	***	***					0.615
16	–	–*		***	***	***			*	***		–*		***	

0.1% $r > 0.554$ significant at 0.1% level; 1% $r > 0.449$ significant at 1% level; 5% $r > 0.349$ significant at 5% level; 10% $r > 0.296$ significant at 10% level

(1) Hatchability (per fertilized egg); (2) Duration of feeding period 5th instar; (3) Duration of feeding period larval stage; (4) Percentage of healthy pupae to the 3rd ecdysed larvae; (5) Amount of reelable cocoons produced per 10,000 rd ecdysed larvae; (6) Cocoon weight; (7) Cocoon shell weight; (8) Percentage of cocoon shell weight; (9) Raw silk percentage; (10) Length of cocoon filament; (11) Weight of cocoon filament; (12) Size of cocoon filament; (13) Reelability percentage; (14) Neatness defects point; (15) Raw silk weight per day of the 5th instar; (16) Pupal weight

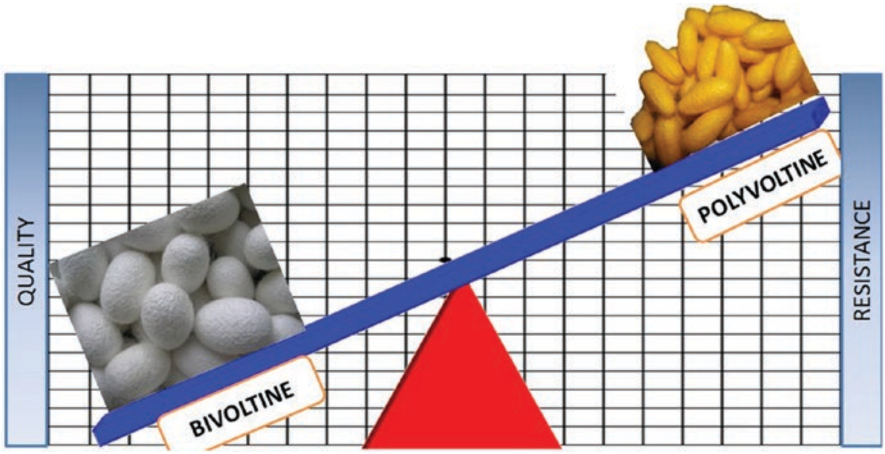


Fig. 4.6 Seesaw balance of quality versus resistance. Yield attributes are linked to the biochemical parameters (Chatterjee et al. 1993)

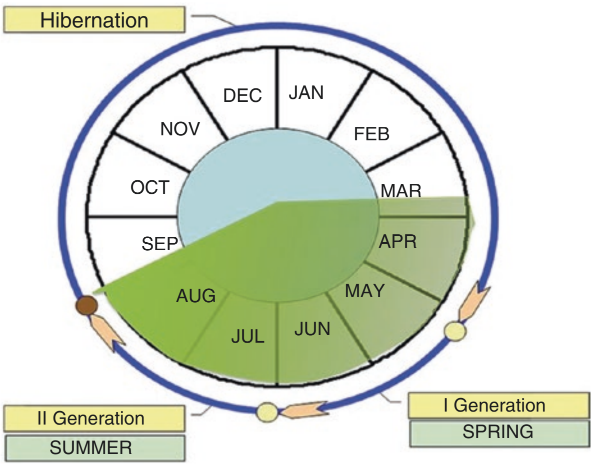


Fig. 4.7 Behavior of bivoltines in temperate regions. They show two successive crops in spring and following summer. Spring crop lays non-diapausing eggs, while summer crop lays diapausing eggs

with the temperature of 25–30°C. They become moths during later half of August and deposit eggs. The laid eggs are brown colored and hibernating (Tazima 1986) (Fig. 4.7).

The freshly laid diapausing eggs also do not have pigments, just like nonhibernating eggs, but after 10–20 days in Japan, eggs become pigmented and turn to purple black. The pigmented eggs stop their development at the age of blastoderm formation and become dormant. Eggs pass through summer, and once they

encounter cold weather of winter for 3–4 months, they continue their development. Hence, "bivoltine" in Japan is alternation of diapausing and non-diapausing generations (Mundkur et al. 2004).

Extensive studies on diapause mechanism are undertaken in temperate conditions, but under tropical conditions, the studies are scanty. In Indian tropical conditions, bivoltines do not behave as they behave in Japanese conditions. They undergo hibernation in every generation. During summer season, sporadic incidences of bivoltines laying non-diapausing eggs are recorded. Conversely, during winter season, sporadic incidences of polyvoltine silkworm races laying diapausing eggs are also recorded. Generally, polyvoltine females lay nondiapausing eggs irrespective of whether they pair with polyvoltine or bivoltine males. Similarly, bivoltine females lay diapausing eggs irrespective of whether they pair with bivoltine or polyvoltine males. Therefore, diapause is a maternally inherited character, and males do not have any role in determining the voltinism of the offspring. However, an exception to this well-established phenomenon was recorded where bivoltine females (which are destined to lay hibernating eggs) lay nonhibernating eggs when they are mated with a special character race, KS-10 males (Mundkur et al. 2004, 2006, 2009, 2010b, c, d). This kind of paternal influence on diapause has opened up many views on the diapause phenomenon in silkworm *Bombyx mori*, from point of view of its expression under tropical conditions.

4.6 Difference Between Diapause and Non-diapause Eggs

Generally there are many differences between diapause and non-diapause type of silkworm eggs (Table 4.4).

4.7 Tropicalization of Temperate Races

A prominent race which supported sericulture industry in South India was C-Nichi. It was evolved from a Chinese × Japanese hybrid (C for Chinese × N for Nippon which means Japan and ichi means one). It lost its hibernating character and much of its productive traits and has become white, highly robust,

Table 4.4 Difference between diapause and non-diapause eggs

Diapausing egg	Non-diapausing egg
Generally eggs are pigmented	Generally non-pigmented
Embryonic development arrested	Continuous development
Diminished egg metabolism	Increased metabolism
Lower oxygen consumption by eggs	Higher oxygen consumption
Preservation of substrates like lipids and carbohydrates is more	Preservation of lipids and carbohydrates is less
Marked resistance to desiccation and low temperature	Sensitive to desiccation and low temperature

low-productive multivoltine race. Likewise an exotic univoltine race got tropicalized and became Boropolu and sustained in West Bengal area. Several attempts were made to import and establish exotic bivoltine or univoltine races, especially from Italy, France, Russia, Japan, China, and Iran. Only a few of these races survived under Indian conditions, especially in Kashmir area, where the climatic conditions are suitable for their survival. In Southern India, which has typical tropical conditions, these bivoltines could not survive for long. Till today Pure Mysore rules the sericulture industry. Likewise in West Bengal, Nistari became the predominant silkworm race.

4.8 Heritability

Most of economic characters are of quantitative type, and each is expressed by cumulative action and interaction of many genes in either an additive or nonadditive mode (HoZoo 1997). Resemblance between relatives and inbreeding depression are two basic genetic phenomena associated with quantitative characters. Resemblance through genetic causes only is defined as heritability. The reverse effect of inbreeding depression is called heterosis or hybrid vigor. In general, the closer the relationship, the closer the resemblance. Selective breeding is based on the resemblance between offspring and parents. The average level of a certain character in the next generation will be improved when the more desirable individuals are used as parents. The extent of resemblance varies with the character. Some characters which show more resemblance are more responsive to selection than others. One characteristic of a population is the degree of resemblance between relatives. A quantitative genetic approach such as heritability estimation will show how the degree of resemblance between different sorts of relatives can be used to predict the outcome of selective breeding. It can also lead a breeder to the best method of selection. Heritability expressed in quantitative terms is the proportion of total variance due to the average effects of genes and also determines the degree of resemblance between relatives. Its predictive role is of important use to breeders, because heritability expresses the reliability of the revealed phenotypic values as a guide to the concealed heritable breeding value of additive gene action or the degree of correspondence between phenotypic value and breeding value. If a breeder chooses parental silkworms according to their phenotypic values, improvement of characters in the subsequent generation can be predicted only by knowing the extent of correspondence between phenotypic values and breeding values. Therefore, the magnitude of heritability is very important as breeders select breeding methods and determine breeding procedures. A distinction is usually made between heritability in "broad" and "narrow" sense, reflecting the components of variation (variance in statistical terms) included in their estimation. Broad sense heritability can be described as the ratio between the genetic variance and phenotypic variance. Narrow-sense heritability is a more meaningful term and thus used almost exclusively by silkworm breeders. It is defined as the ratio of additive genetic variance to total phenotypic variance.

Heritability is a property of a certain character as well as of the population and of the environment to which the individual silkworms are subjected. The value of heritability varies depending on the magnitude of all the components of variance. A change in any one of these components will therefore influence heritability estimates. All the genetic components are influenced by gene frequencies, which may be different from one population to another, according to their history. Large populations will show higher heritability than small populations, in which a significant amount of fixation has occurred for some time. More variable environmental rearing conditions are expected to reduce heritability, while more uniform conditions will increase it. Whenever a value is stated for the heritability of a given character, it refers to a particular population under particular conditions. A considerable range of variation is thus commonly shown among different estimates of heritability for the same character, although it may be partly due to statistical sampling. The greater the heritability value, in general, the smaller the variation of the character because of environmental differences.

The relatively high values of h that were found out for some leading selection traits such as cocoon shell weight, filament length, etc., are informative for the big genetic variation in populations of *Bombyx mori L.* and for the perspectiveness of phenotype selection. In the same time, however, the relatively low values of h for the trait number of eggs in one batch determine the considerably trait magnitude by the environmental conditions (Singh et al. 1992b). Traits with low h can be improved by the inclusion of other closely correlated traits with relatively high values of h in the breeding programs. Relatively higher values of heritability were found for cocoon weight (73.60%), pupal weight (78.50%), shell weight (80.20%), growth rate (79.30%), raw silk yield (79.00%), and shell ratio (72.40%) (Chatterjee and Datta 1992; Singh and Singh 1993; Singh 1994; Singh et al. 1994; Yan 1983; Rajanna and Reddy 1990a, b). Middle values for larval period duration were found, while low heritability was detected for the traits pupation rate (28.00%) and reelability (19.00%) (Gamo and Hirabayashi 1983) (Table 4.5).

4.9 Hybrid Vigor and Environment

Hybrid vigor refers to positive heterosis. It is the extra vigor or improvement in performance shown by the F1 progeny over either of its parents or mid-parent value. Hybrid vigor is the manifestation of interaction between the genes or alleles of two pure lines. However the phenotypic expression is always dependent on the interaction between genotype and environment. Even if the organism possesses a very good genetic makeup, the phenotype is the result of its interaction with the environment. Thus, for optimum expression of the genotype, congenial environment is necessary.

The phenotypic expression is also dependent upon the genetic plasticity or buffering capacity of the organism. The extra vigor shown by the outcross between two inbred lines of silkworm could be due to the extra buffering capacity of the resultant organism.

Table 4.5 Heritability of some economic traits in *Bombyx* mori

Characters	Heritability
Cocoon weight	73.60
Pupal weight	78.50
Cocoon-shell ratio	72.40
Pupation rate	19.00
Shell weight	80.20
Growth rate	79.30
Raw silk %	79.00
Reelability	28.00

Source: Tribhuwan Singh et al. (2011)

4.10 Theories on Breeding

There are many theories of breeding methodology to make the breed best "fit" to the given environment.

4.10.1 Ideal Environment Breeding

Falconer (1952) advocates that "the character required is best selected for under environmental conditions which favour its fullest expression. Once developed, other characters specially required for new environment also will be present in such animals." Many silkworm breeders subscribe to this theory (Toyama 1906a, b, 1913).

4.10.2 Native Environment Breeding

The high-yielding silkworm breeds developed through inbreeding of hybrids by selection for various attributes under laboratory conditions (Krishnaswami 1978). The silkworm races thus developed are utilized to obtain polyvoltine × bivoltine crossbreeds or bivoltine × bivoltine hybrids for commercial usage. The farmers often fail to produce the optimum conditions required by these combinations. As a result, only 40% of the yield attributes are expressed in the field (Nagaraju 1998). Falconer (1952, 1960, 1981) advocates "performance is best improved by selection under the conditions in which the performance is subsequently measures." He explains that quantitative traits or metric traits show continuous variation because of $P = G \times E$ effect. Therefore, the segregation of genes cannot be followed individually (Falconer 1981). Many tropical breeders subscribe to this theory (Rajanna and Reddy 1990a, b, 1998).

4.10.3 Shuttle Breeding

High-yielding breeds bred in laboratory have the limitations of limited population rearing. Chatterjee (1993) supported the breeding plan of taking breeding materials

during F10 to the different Regional Research Stations so that they are exposed to various climates. Then they are brought back to the main breeding laboratory for applying appropriate selection pressure.

4.10.4 Voltinism Breeding

Discovery of *pnd* and *npnd* genes and their behavior in tropical environment lead to this special branch of breeding (Chatterjee 1993; Murakami 1986, 1988, 1989; Murakami and Ohtsuki 1989; Subramanya and Murakami 1994). Murakami (1986) showed that npnd (nonpigmented non-diapausing) is a sex-linked gene and responsible for multivoltinism exhibiting maternal inheritance. He also revealed that npnd is epistatic to pnd (pigmented non-diapausing) which was described by Katsumata (1968).

4.10.5 Avoltinism Breeding

The tropical bivoltine and multivoltine race exhibit distinct economic advantages. Survival is generally attributed to multivoltines and productivity to bivoltines. Voltinism remains a barrier and bottleneck in balancing and harvesting these two critical attributes. Discovery of new dominant voltinism gene “Id” has become a new tool in silkworm breeding. The presence of Id gene overcomes the effect of voltinism. It can be transduced to the desired race thereby making it a “voltine-less” race. A new terminology is coined for such character, “avoltinism,” which means voltinism-less or “without voltinism.” This tool holds the promise for breaking the inverse relationship and balancing the polyvoltine survival traits with bivoltine quantitative and qualitative traits (Mundkur et al. 2004, 2010a, b, c, d, 2011, 2012).

4.10.6 Biotechnology, Genomics, and Marker-Assisted Selection Breeding

Review of progress in applying molecular genetic and genomic technologies to studies in the domesticated silkworm, *Bombyx mori*, highlighting its use as a model for Lepidoptera, and in sericulture and biotechnology has been done by many scientists (Nagaraju 1999; Nagaraju et al. 2001; Goldsmith et al. 2005). Dense molecular linkage maps are being integrated with classical linkage maps for positional cloning and marker-assisted selection. Classical mutations have been identified by a candidate gene approach. Cytogenetic and sequence analyses show that the W chromosome is composed largely of nested full-length long terminal repeat retrotransposons. Z-chromosome-linked sequences show a lack of dosage compensation. The downstream sex differentiation mechanism has been studied via the silkworm homologue of double-sex. Expressed sequence tagged databases have been used to discover

Lepidoptera-specific genes, provide evidence for horizontal gene transfer, and construct microarrays. Physical maps using large-fragment bacterial artificial chromosome libraries have been constructed, and whole-genome shotgun sequencing is underway. Germline transformation and transient expression systems are well established and available for functional studies, high-level protein expression, and gene silencing via RNA interference.

4.11 Understanding Tolerance and Resistance in Silkworm Breeds

Resistance is an absolute term where the individual completely immunizes itself to a particular stress. This kind of situation is observed in case of biotrophic pathogen (pathogen needing a live host to draw nutrient) infection when the host contains a resistance gene and the infecting pathogen contains its corresponding a virulence gene. Resistance can be summarized as the ability of the organism to limit pathogen growth and infection.

Tolerance is rather a relative term, and it is also man made to some extent. Tolerance does not limit infection but instead reduces or offsets its negative fitness consequences. Generally biotic stress challenges the resistance of the individual, and abiotic stress challenges the tolerance and resistance, and tolerance may provide equivalent short-term benefits but have fundamentally different epidemiological consequences and thus exhibit different evolutionary behaviors (Felix Horns and Hood Michael 2012).

The defense reaction in silkworm against bacteria is the production of antibacterial protein in the hemolymph. The defense reaction in silkworm against nuclear polyhedrosis virus (NPV) includes (1) antiviral substance in the gut juice of the silkworm (Aizawa 1962, 1991) and (2) viral inhibitory factors (VIF) produced in the silkworm hemolymph (Hayashita et al. 1968). Red fluorescent protein (RFP) also shows profound antiviral activity.

Conclusion

Silkworm breeders of Indian tropical conditions are trying very best for the substantial improvement in the cocoon yield, silk recovery, and quality in the silkworm breeds they develop, through the integration of all the available approaches which include conventional as well as molecular biology tools. Foregoing discussions clearly indicate that the environment is the critical limiting factor, which include biotic and abiotic factors, which determine the final performance of the developed breeds. Southern zone of India is characterized by semiarid areas by tropical conditions, often experiencing deficit rainfall and high temperature, with higher biotic as well as abiotic stress. Natural selection for this area is the polyvoltine breed with moderate production with higher survival rates. Balanced increase in productivity, quality, and survivability is possible by considering all the relevant factors in breeding.

References

Aizawa K (1962) Antiviral substance in the gut juice of the silkworm, *Bombyx mori* (L). J Insect Pathol 4:72–76

Aizawa K (1991) Defense reactions of the silkworm, *Bombyx mori* (L), against the nuclear polyhedrosis. Sericologia 31:201–203

Allen Orr A (2009) Fitness and its role in evolutionary genetics. Nat Rev Genet 10(8):531–539

Andrewartha MG (1952) Diapause in relation to the ecology of insects. Bio Rev 27:50–107

Barlow HS (1982) An introduction to the moths of South East Asia. Malayan Nature Society, Kaulalumpur, pp 1–305

Chatterjee SN (1993) Silkworm breeding in India. Sericologia 33(3):427–447

Chatterjee SN, Datta RK (1992) Hierarchical clustering of 54 races and strains of the mulberry silkworm, *Bombyx mori* L: significance of biochemical parameters. Theor Appl Genet 85(4):394–402

Chatterjee SN, Rao CGP, Chatterjee GK, Ashwath SK, Patnaik AK (1993) Correlation between yield and biochemical parameters in the mulberry silkworm, *Bombyx mori* L. Theor Appl Genet 87(3):385–391

Falconer DS (1952) The problem of environment and selection. Am Nat 86:293–298

Falconer DS (1960) Introduction to quantitative genetics. Oliver & Boyd, Edinburgh

Falconer DS (1981) Introduction to quantitative genetics, 2nd edn. Longman, London

Felix Horns F, Hood Michael E (2012) The evolution of disease resistance and tolerance in spatially structured populations. Ecol Evol 2(7):1705–1711

Gamo T, Hirabayashi T (1983) Genetic analysis of growth rate, pupation rate and some quantitative characters by diallel cross in the silkworm, *Bombyx mori*. Jpn J Breed 33:178–190

Gamo T, Ohtsuka Y (1980) Phylogenetic studies on the racial differences of the silkworm, *Bombyx mori*, on the basis of polymorphic genes in haemolymph proteins. Bull Sericult Exp Sta 28:15–50

Goldsmith MR, Shimada T, Abe H (2005) The genetics and genomics of the silkworm, *Bombyx mori*. Annu Rev Entomol 50:71–100

Gowda BLV, Narayanaswamy TK, Munirajappa R (1988) Impact of pupal weight on growth and development of the following generation in the silkworm Indian race NB7 (*Bombyx mori*). Sericologia 29:481–489

Gowda BLV, Sannaveerappanayar VT, Shivayogeshwar B (1989) Fecundity and hatchability in mulberry silkworm, *Bombyx mori* (L.) as influenced by pupal weight. International congress on tropical sericultural practices, part VI, Bangalore, 18–23 February 1989, pp 21–24

Grekov D, Petkov N (1990) Correlation between some traits and possibilities for prognostication heterosis of F1 crosses on hybridization of *Bombyx mori* L. Zhivotnov's Nauki 27:80

Hayashita K, Nishida J, Matsubara F (1968) Inactivation of nuclear polyhedrosis virus in the digestive juice of silkworm, *Bombyx mori* (L). I. Comparison of antiviral activities in the digestive juice of larvae reared on natural and artificial diets. Jpn J Appl Entomol Zool 12:189–193

HoZoo L (1997) Principles and techniques of silkworm breeding. ESCAP, United Nations, New York

Jayaswal KP, Singh T, Sen SK (1990) Correlation between some economic parameters and their application in silkworm breeding. Indian Silk 29:25–27

Jayaswal KP, Singh T, Subba RG (1991) Effect of female pupal weight on fecundity of mulberry silkworm *Bombyx mori*. Indian J Seric 30:141–143

Jonaka N (1986) Some parameters of basic quantitative breeding characters in silkworm, *Bombyx mori*. II. Correlations between the basic breeding characters. Genet Sel 19:144–150

Katsumata F (1968) Non maternal inheritance in Voltinism observed in the crossing experiments between Indonesian polyvoltine and Japanese bivoltine races of silkworm, *Bombyx mori*. J Seric Sci Jpn 37:453–461

Krishnaswami S (1978) New technology of silkworm rearing. Bulletin No. 2. Central Sericultural Research and Training Institute, Mysore, Central Silk Board, India, pp 1–23

Liu Q-X, He S-M (1991) Studies on the efficiency of selection for improving technological characters of cocoon filament of *Bombyx mori* L. Canye Kaxue 17:75–79

Long NV, Petkov N (1987) Breeding—genetic studies in some silkworm (*Bombyx mori*) breeds. I. Variability and correlations of quantitative characters. Genet Sel 20:58–62

Miyahara T (1978) Selection of long filament length basic variety. I. Effect of selection in the later generations. Acta Sericologia Sinica 106:73–78

Morohoshi S (2000) Development physiology of silkworms. In: Translation of second Japanese edition of Kaiko no Hatsuiku Seiri by Morohoshi S, (1979). Oxford and IBH Pub.Co.Pvt.Ltd, New Delhi

Mundkur R, Murthy M, Raghuraman MR, Bongale UD (2004) Prevention of diapause in bivoltine eggs of the silkworm, *Bombyx mori* L., through a cross with the race KS-10 as male parent. Int J Ind Entomol 9(1):107–109

Mundkur R, Murthy M, Rao SK, Raghuraman R, Bongale UD (2006) Paternal inheritance of voltinism: a new tool to understand the gene regulation in the silkworm, *Bombyx mori*. In: Proceedings of international symposium on insect genetics and genome, CDFD, Hyderabad, 9–11 Jan 2006

Mundkur R, Murthy M, Latha R, Krishna Rao S, Sekharappa BM (2009) Indication of expression of inhibitor of diapauses gene in silkworm, *Bombyx mori*. In: National conference on recent trends in animal physiology, Manasa Gangotri, Mysore, 29–30 Oct 2009

Mundkur R, Katti SR, Chandrakala MV, Sekharappa BM (2010a) Diapause in silkworm, *Bombyx mori* L with special reference to the paternal influence: a review. Sericologia 50(2):145–170

Mundkur R, Murthy M, Latha R, Krishna Rao S, Sekharappa BM (2010b) Avoltinism breeding: a new concept in silkworm breeding. In: Silkworm breeder's meet, RSRS Coonoor, 2–3 Dec 2010

Mundkur R, Murthy M, Latha R, Krishna R, Sekharappa BM (2010c) "Id" inhibitor of diapauses, a new gene as diapauses switch in silkworm, *Bombyx mori*. In: Nattional seminar on emerging trends in animal biotchnology, Kakatiya University, Warangal, 26–27 Mar 2010

Mundkur R, Murthy M, Latha R, Rajanna GS, Krishna Rao S (2010d) Reporting of a new gene inhibitor of diapause(Id) in the silkworm, *Bombyx mori* L. Sericologia 50(3):359–364

Mundkur R, Murthy M, Krishna Rao S (2011) Modification of diapause induced by inhibitor of diapause gene (Id), after fertilization in silkworm, *Bombyx mori*. In: International conference on life science research for rural and agricultural development, CPRS, Patna, Bihar, 27–29 Dec 2011

Mundkur R, Murthy M, Krishna Rao S (2012) Termination of diapause by Inhibitor of diapause gene (Id) after fertilization in silkworm, *Bombyx mori*. In: National seminar on advances in zoology and life processes, Goa University, 9–11 Feb 2012

Murakami A (1986) Changes in embryonic diapause in the tropical multivoltine silkworm stock Cambodge. In: Abstract: 34th Annual meetings of the Tokyo district Sericul. Sci Soc 27

Murakami A (1988) Ecogenetical studies on tropical mulberry *Bombyx mori*. In: Proceedings of the international congress on tropical sericulture practices, Bangalore, India, pp 11–23

Murakami A (1989) Genetic studies on tropical races of silkworm (*Bombyx mori*) with special reference to cross breeding strategy between tropical and temperate races. 2. Multivoltine silkworm strains in Japan and their origin. JARQ 23:123–127

Murakami A, Ohtsuki Y (1989) Genetic studies on tropical races of silkworm, *Bombyx mori*, with special reference to cross breeding strategy between tropical and temperate races. 1. Genetic nature of the tropical multivoltine strain, Cambodge. JARQ 23:37–45

Nagaraju J (1998) Silk yield attributes and complexities. In: Reddy GS (ed) Silkworm breeding. Oxford Press, New Delhi, pp 168–185

Nagaraju J (1999) Biotechnology: a novel concept for silkworm improvement. In: Devaiah MC, Narayanaswamy KC, Maribashetty VG (eds) Advances in mulberry sericulture. C.V.G. Publications, Bangalore, pp 208–242

Nagaraju J, Klimenko V, Couble P (2001) The silkworm, *Bombyx mori*: a model genetic system. In: Reeve ECR (ed) Encyclopaedia of genetics. Fitzroy Dearborn Publishers, London, pp 219–239

Ozdzenska B, Kremky J (1987) Estimation of heritability and genotypic, phenotypic and environmental correlations in out-bred populations of mulberry silkworm, *Bombyx mori* L. Sericologia 27:633–638

Petkov N (1981a) The possibility of forecasting the effectiveness of selecting lines of the silkworm, Bombyx Mori based on weight and silk content of cocoons. Selskotopanska Nauka 19:92–96

Petkov N (1981b) Variability and correlations between some characteristic features of silkworm (*Bombyx mori* L). Zhivotnov'd Nauki 18:83–86

Petkov N (1981c) Phenotypic correlations and regressions between some silkworm (*Bombyx mori*) breeding characters. Genet Sel 14:386–390

Rajanna GS, Reddy GS (1990a) Studies on the variability and interrelationship between some quantitative characters in different breeds of silkworm, *Bombyx mori* L. Sericologia 30:67–74

Rajanna GS, Reddy GS (1990b) New dimensions in tropical silkworm race breeding. Indian Silk 28:8–12

Rajanna GS, Reddy GS (1998) New approaches to silkworm improvement aimed at narrowing yield gaps. In: Reddy GS (ed) Silkworm breeding. Oxford Press, New Delhi, pp 168–185

Satenahalli SB, Govindan R, Goud JV, Magdum SB (1990) Genetic parameters and correlation coefficient analysis in silkworm, *Bombyx mori*. Mysore J Agric Sci 24:491–495

Singh T (1994) Correlation between pupal weight and fecundity in *Bombyx mori* (L). Ann Entomol 12:5–7

Singh T, Singh K (1993) Heritability and correlations between some economic characters in *Bombyx mori* (L). Sci Cult 59:51–52

Singh T, Jayaswal KP, Subba RG (1992a) Correlation studies between some breeding parameters of silkworm, *Bombyx mori* (L). J Zool Res 5:47–50

Singh T, Singh K, Das M (1992b) Astounding effect of correlated parameters in silk industry. Indian Text J 102:26–30

Singh T, Jayaswal KP, Subba RG (1992c) Correlation studies between some breeding parameters of silkworm, *Bombyx mori* (L). J Zool Res 5:47–50

Singh T, Chandrashekharaiah A, Samson MV (1994) Selection strategies in relation to correlation and heritability in the silkworm, *Bombyx mori* L. Bull Seric Res 5:37–41

Singh T, Bhat MM, Khan MA (2011) Critical analysis of correlation and heritability phenomenon in the silkworm, *Bombyx mori* (Lepidoptera: bombycidae). Adv Biosci Biotechnol 2(5):347–353

Sonobe H, Odake H (1986) Studies on embryonic diapause in the pnd mutant of the silkworm, *Bombyx mori*; V. Identification of a pnd+ gene-specific protein. Rouxs Arch Dev Biol 195:229–235

Subramanya G, Murakami A (1994) Climatic differential phenotypic expression of voltine genes in *Bombyx mori* L. Indian J Seric 33(2):103–109

Tazima Y (1986) The genetics of the silkworm. Logos Press, Academic Press, London

Toyama K (1906a) Mendel's law of heredity as applied to the silkworm crosses. Biol Zentralbl 26:321–334

Toyama K (1906b) Breeding methods of silkworm. Sangyo Shimpo 158:282–286

Toyama K (1913) Maternal inheritance and mendelism. J Genet 2:351–405

Yan LL (1983) The estimates of heritability of pupal weight, cocoon weight and number of eggs laid in the silkworm (*Bombyx mori*) and its genetic correlation between these characters and path coefficient analysis. J Seric Sci China 9:149–155

Yoshitake N (1968) Phylogenetic aspects on the origin of Japanese race of the ilkworm, *Bombyx mori* L. J Sericol Sci Jpn 37:83–87

Long Noncoding RNA: Disclosing New Horizon in the Molecular World of Insects

5

Dhiraj Kumar, Xiaolong Hu, Rui Guo, Renyu Xue, Guangli Cao, and Chengliang Gong

Abstract

Long noncoding RNAs (lncRNAs) are the most versatile group of nonprotein-coding RNAs consisting of nucleotides of length more than 200 bp. Similar to mammal's genome phenomenon, thousands of lncRNAs have been discovered in the insect's genome through RNA sequencing technology and computational methods, which contributes to the diverse biological processes including diseases to regulate the gene expression, dosage compensation, and epigenetic imprinting of entire chromosome. In fruit fly, lncRNAs exposed noteworthy functions in the behavioral processes, sex, and neural development. However, in the silkworm, lncRNAs were linked with silk synthesis and affect the apoptosis; additionally, other baculoviral lncRNAs contributed to establishing the complex regulation of viral gene expression in baculovirus-infected BmN cells. In diamondback moth, lncRNA gene expression study revealed the insecticidal resistant activity, whereas caste differentiation and behavior mechanism in honeybee were also significantly investigated. Therefore, lncRNAs exist in various insect's genomes, opening a new horizon for biotechnologist to identify, study, and disclose the gene expression, regulatory and biological functions of lncRNAs in insects.

D. Kumar (✉) • X. Hu • R. Xue • G. Cao • C. Gong (✉)
Molecular Biology Laboratory, School of Biology and Basic Medical Science, Soochow University, Suzhou 215123, Jiangsu, China
e-mail: drkumarindia@yahoo.com; gongcl@suda.edu.cn

R. Guo
College of Bee Science, Fujian Agriculture and Forestry University, Fuzhou 350002, China

D. Kumar, C. Gong (eds.), *Trends in Insect Molecular Biology and Biotechnology*, https://doi.org/10.1007/978-3-319-61343-7_5

5.1 Introduction

In the past, RNA fit cleanly into the central dogma as a messenger between DNA and protein. RNA molecules have frequently emerged as vibrant and resourceful regulators of the genome. However, several scientists have identified a different kind of RNA which does not participate in the protein synthesis, the so-called dark matter of genome (Kapranov and Laurent 2012). In eukaryotes, despite the fact that noncoding DNA has been frequently named as “junk DNA” with no evolutionary imperative, it encounters natural selection (Ponting et al. 2009). A little information is accessible about the origin and advancement of lncRNAs compared with the protein-coding gene. LncRNAs display a low conservation of sequence and rapid evolution among mammals (Kutter et al. 2012). In the last decades, bioinformatics and RNA sequencing technologies have been proven as influential and significant tools to explore whole genome sequencing including lncRNA. In human and mouse transcriptome analysis, about 58,000 and 8000 expressed genes were, respectively, classified as lncRNAs (Iyer et al. 2015; Sun et al. 2013) and present at a specific time-point in a particular cell type or tissue (Wang et al. 2009; Djebali et al. 2012). Just a few years back, specific consideration has been paid to the class of long noncoding RNAs (lncRNAs) as they have been associated with different systems, for example, cis- and trans-control of interpretation, dosage remuneration, engraving and contending endogenous RNA (Ulitsky and Bartel 2013; Fatica and Bozzoni 2013; Gardini and Shiekhattar 2014; Bonasio and Shiekhattar 2004), and their diverse roles in a variety of critical biological processes in higher organisms (Eddy 2001). Long noncoding RNA (lncRNA), which is the most versatile group of RNA consisting of more than 200 bp without a coding ORF (Kapranov et al. 2007), emerges as potent regulator of the gene expression and dramatically altered our understanding of the cell biology in diseases. The majority of the known miRNAs are exceptionally rationed crosswise over different life-forms from insect to human (Lim et al. 2003a, b). Similar to mammal genome phenomenon, in recent years, through computational methods and RNA sequencing technology, thousands of lncRNAs have been discovered in the insect’s genome. The main function of lncRNA in regulation is to control the expression of distinct genes. As lncRNAs are less conserved in the nucleotide sequence across phylogenetically related species, therefore it is challenging to identify lncRNAs by general sequence searching (Necsulea et al. 2014). LncRNAs are homologous to mRNAs in terms of posttranscriptional modifications such as splicing, polyadenylation, and capping (Derrien et al. 2012). Nevertheless, lncRNAs are less conserved comparatively in human and other vertebrates. Thousands of lncRNAs have been discovered in the genome of *Drosophila melanogaster*, *Anopheles gambiae*, *Apis mellifera*, and *Bombyx mori* (Li et al. 2014; Zhang et al. 2014; Pauli et al. 2012; Wang et al. 2015; Hao et al. 2015; Young et al. 2012; Padron et al. 2014; Jayakodi et al. 2015; Jenkins et al. 2015; Liu et al. 2012; Wu et al. 2016). Initially, Wahlestedt (Wahlestedt 2013) described in details about the formation of lncRNA (Fig. 5.1) including various linked processes such as translation and transcription. He also explained about how lncRNA developed and played a significant role in various biological functions.

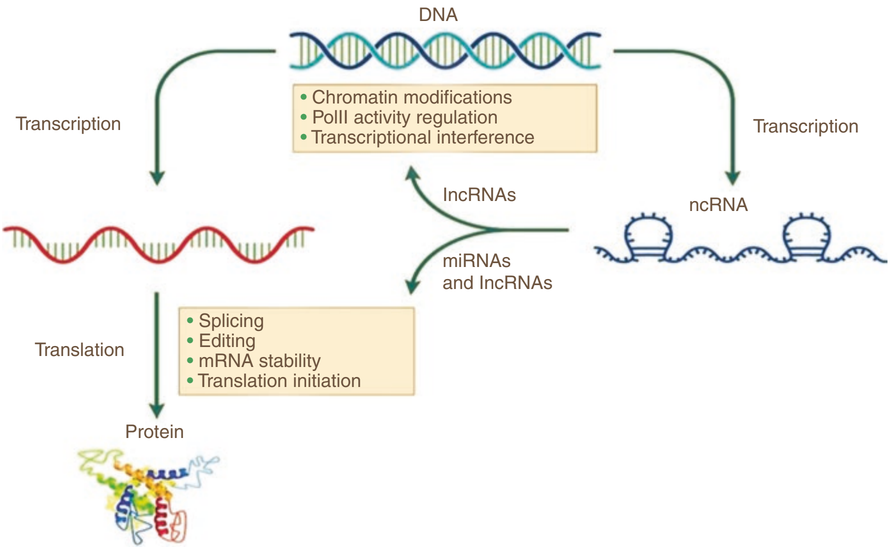


Fig. 5.1 Formation of lncRNA (Wahlestedt 2013)

The fruit fly has been one of the globally exploited and first-choice research model organism for genetics research. The fast extension of genome sequence data of *Drosophila* has led to the emergence of comparative genomics protocols to investigate the genome-wide alignment. There are about 40,000 introns with sequence length, accounting for 22% of the entire *D. melanogaster* genome, which is a little bit larger than 19% of the total coding sequences (Adams et al. 2000; Holt et al. 2002).

5.2 Discovery of lncRNAs in Insects

After the discovery of lncRNA in mammals and other vertebrates, researchers started giving attention toward investigation of lncRNA in insects and its possible biological functions. The first breakthrough was recorded in *D. melanogaster*; about 4000 candidate lncRNAs (Brown et al. 2014) have been identified through RNA-Seq technology. Afterward several lncRNAs were investigated in other insects namely *A. gambiae* (Jenkins et al. 2015), *A. mellifera* (Jayakodi et al. 2015), and *B. mori* (Wu et al. 2016). The biological functions of few identified genes, namely, lncRNA CRG (Li et al. 2012), male-specific lncRNAs roX1 and roX2 (Deng and Meller 2006), Sphinx lncRNA (Chen et al. 2011), and yellow-achaete intergenic RNA (yar) lncRNA (Soshnev et al. 2011), were studied in *D. melanogaster*, whereas 5,383,727 and 1382 lincRNAs have been experimentally confirmed in *Plutella xylostella* (Etebari et al. 2015) including lncRNAs Nb-1, Ks-1, AncR-1, kakusei, lncov1, and lncov2 of *A. mellifera* (Kiya et al. 2008a; Kiya et al. 2012; Tadano et al. 2009; Sawata et al. 2002; Sawata et al. 2004; Humann and

Hartfelder 2011). Various noncoding RNAs' biological functions have been well studied in *B. mori* such as PiWi-interacting RNAs (Kawaoka et al. 2011), microRNAs (Jia et al. 2015), and snoRNA (Li et al. 2014). However, lncRNAs functionally remain poorly classified in the silkworms except for lncRNA Fben-1 (Taguchi et al. 2011) which is expressed in the female brain. In the last few years, the role of various lncRNAs (lncRNA-p21, lncRNA-GAS5, lncRNA UCA1) in apoptosis was also examined in humans (Tran et al. 2015; Shi et al. 2015; Wu et al. 2013). In the case of invertebrates, two Bcl-2 family proteins homologous to Bok (a mammalian pro-apoptotic member) in *D. melanogaster* were successfully recognized (Tatsushi and Masayuki 2004). After identification of Bcl-2 gene, functional analysis of numerous lncRNAs has been discovered in *D. melanogaster* (Smith et al. 2001). Afterward, Pan (Pan et al. 2010) studied that *BmBuffy* played a significant role in hydroxycamptothecin-induced apoptosis in BmN-SWU1 cells of *B. mori* and possesses potential apoptosis-related genes which give an important evidence to investigate the role of lncRNA in apoptosis. Systematic identification of lncRNAs (Wu et al. 2016) in the few tissues of *B. mori* was disclosed in the presence of long nonprotein-coding RNAs in the genome of the silkworm; nonetheless, studies related to gene expression and biological function including apoptosis are still scanty and need further investigation. Our research group also identified a number of lncRNAs in the silkworm genome and is studying the lncRNAs' gene functions in the silkworm.

5.3 Classification of lncRNAs

According to an important report of Kowalczyk and Higgs (Kowalczyk et al. 2012), they made an effort to the insight of various researcher findings and revealed that nonprotein-coding gene has been divided into noncoding RNA and long noncoding RNA. They also explained about the number of known transcripts and transcript length. On that basis, ncRNA (miRNA, snoRNA, snRNA, piRNA, tRNA) and lncRNAs (antisense ncRNA, enhancer ncRNA (eRNA), enhancer ncRNA (meRNA) II, intergenic ncRNA, pseudogene ncRNA) have been categorized (Fig. 5.2 and Table 5.1).

5.4 How lncRNA Differ from mRNA?

Generally, a little extent of lncRNAs has so far been explored, and despite the fact, this is predominantly because it doesn't look like protein-coding gene, whose succession themes and sequence motifs are symbolic of their function; lncRNA sequences are not usually conserved and they don't tend to contain conserved motif. The characteristics of lncRNA and mRNA are explained in Table 5.2.

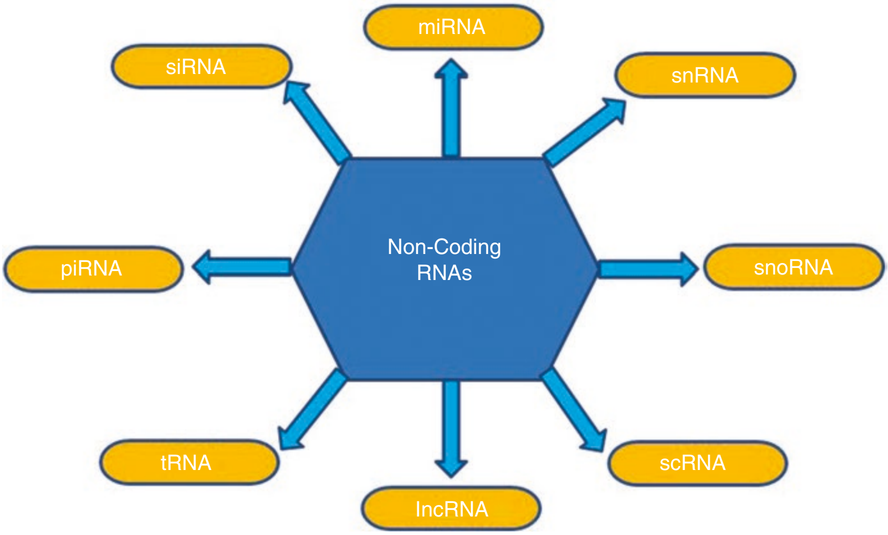


Fig. 5.2 Diversity of noncoding RNAs

Table 5.1 Types of noncoding and long noncoding RNAs and their transcripts

ncRNA	No. of known transcripts	Transcript lengths (nucleotides: nt)
Precursors to short RNAs		
snRNA	1944	1000
miRNA	1756	>1000
snoRNA	1521	>100
tRNA	497	>100
piRNA	89	Unknown
Long ncRNAs		
Intergenic ncRNA	6742	102–105
Antisense ncRNA	5446	100–>1000
Enhancer ncRNA (eRNA)	>2000	>1000
3′ UTR ncRNA	12	>100
Pseudogene ncRNA	680	102–104

LncRNAs can be classified into the following locus biotypes based on their genome location with respect to protein-coding genes (Ponting et al. 2009; Mercer et al. 2009) (Figs. 5.3 and 5.4).

Intergenic lncRNA: Intergenic lncRNAs are transcribed intergenetically from both strands.

Table 5.2 Gray and cyan between lncRNA and mRNA (courtesy: Exon)

lncRNA	mRNA
Tissue-specific expression	Tissue-specific expression
Form secondary structure	Form secondary structure
Undergo posttranscriptional processing, i.e., 5′ cap, polyadenylation, splicing	Undergo posttranscriptional processing, i.e., 5′ cap, polyadenylation, splicing
Important roles in diseases and development	Important roles in diseases and development
Nonprotein-coding, regulatory functions	Protein-coding transcript
Poorly conserved between species	Well conserved between species
Many predominantly nuclear, others nuclear and/or cytoplasmic	Present in both nucleus and cytoplasm
Currently ~30,000 lncRNA transcripts, predicted 3–100-fold of mRNA in number	Total 20–24,000 mRNAs
Expression level: very low to moderate	Expression level: low to high

Table 5.3 LncRNAs functions in insect model *D. melanogaster* and *A. mellifera* (Fabrice and Thomas 2015)

Gene	Species	Function	Mode
Gene regulation hsr-v	*D. melanogaster*	Long noncoding RNAs produced by the hsw-v gene are actively expressed in nuclei, forming spots called perinuclear omega speckles, in response to heat shock stress. These speckles are involved in the redistribution and sequestration of multiple processing proteins, in particular, heterogeneous nuclear ribonucleoproteins (hnRNPs), HP1, or polII, which strongly affect multiple cellular networks subsequent to a stress (Lakhotia et al. 2012; Lakhotia 2011)	Trans
Epigenetics control of gene regulation rox1/rox2	*D. melanogaster*	In X0 male *Drosophila*, the transcription of genes located on the X chromosome is increased relative to the level of XX females by a mechanism known as dosage compensation. This mechanism is connected to the acetylation of histone H4 at lysine 16 induced by a protein complex which involves two long noncoding RNAs roX1 and roX2 (Deng and Meller 2006; Smith et al. 2001)	Trans

Table 5.3 (continued)

Gene	Species	Function	Mode
Development bithorax	*D. melanogaster*	The bithorax complex (BX-C) plays a key role in *Drosophila* development and covers a region larger than 300 kb that includes only four protein-coding genes. Noncoding genes have already been shown to be concomitantly expressed from the BX-C domain to regulate in cis the BX-C proteins in specific abdominal segments (Lipshitz et al. 1987; Pease et al. 2013)	Cis
Behavior yar	*D. melanogaster*	Yar is a long noncoding RNA, highly expressed during embryogenesis and located in a neural gene cluster between yellow (y) and achaete (ac) (Soshnev et al. 2008). With the help of mutants, the function of this long noncoding RNA has been recently refined as a regulator of y and ac transcription, affecting as well the sleep behavior of the *Drosophila* (Soshnev et al. 2011)	Cis
Sphinx	*D. melanogaster*	Sphinx is a lncRNA involved in the regulation of male courtship behavior. Sequence variations between close *Drosophila* species advocate for a functional role and a rapid adaptation. The mutagenesis of sphinx in *Drosophila* reveals the emergence of male-male courtship behavior, probably by the disruption of some sensory circuits, which is supported by its specific expression in chemosensory organs (Chen et al. 2011)	Unknown
Nb-1	*A. mellifera*	Nb-1 is a 700 nt transcript, whose longest ORF encodes a putative 32 amino acid without any sequence conservation. This lncRNA is expressed in the honeybee brain with variation according to the age of the colony workers testifying to its putative role in polyethism (Tran et al. 2015)	Unknown

(continued)

Table 5.3 (continued)

Gene	Species	Function	Mode
Neural expression Ks-1	*A. mellifera*	Sawata et al. identified a 17knt transcript that is expressed restrictively in the mushroom body of Kenyon cells in the honeybee brain and which accumulates in the nucleus. The transcript exhibits seven putative ORFs longer than 67 amino acids without any conservation in a related species (*Apis cerana*) nor similarity with known proteins (Sawata et al. 2002)	Unknown
AncR-1	*A. mellifera*	AncR-1 is preferentially expressed in the brain, sexual tissues, and some secretory organs and accumulates in nuclei. It contains multiple alternate isoforms, which are derived from a 6.9 kbp genomic locus (Sawata et al. 2004)	Unknown
Kakusei	*A. mellifera*	The kakusei is a 7000 nt long noncoding RNA with multiple constitutive and inducible variants, the expression of which is transiently upregulated by neural activity. It is localized exclusively in neural nuclei in discrete nuclear compartments. This gene may play specific roles in RNA metabolism in the honeybee brains, irrespective of behavioral experience (Kiya et al. 2008b)	Unknown
Lnccov1/lnccov2	*A. mellifera*	These two transcripts lack evidence of functional ORFs and are differentially expressed in queen and worker ovariole transcriptomes at the embryonic stage. Temporal expression shows that lnccov1 might be involved in the autophagic cell death of ovarioles during worker embryogenesis, and fluorescent in situ hybridization (FISH) indicates perinuclear localization in omega speckle-like structures (Humann and Hartfelder 2011)	Unknown

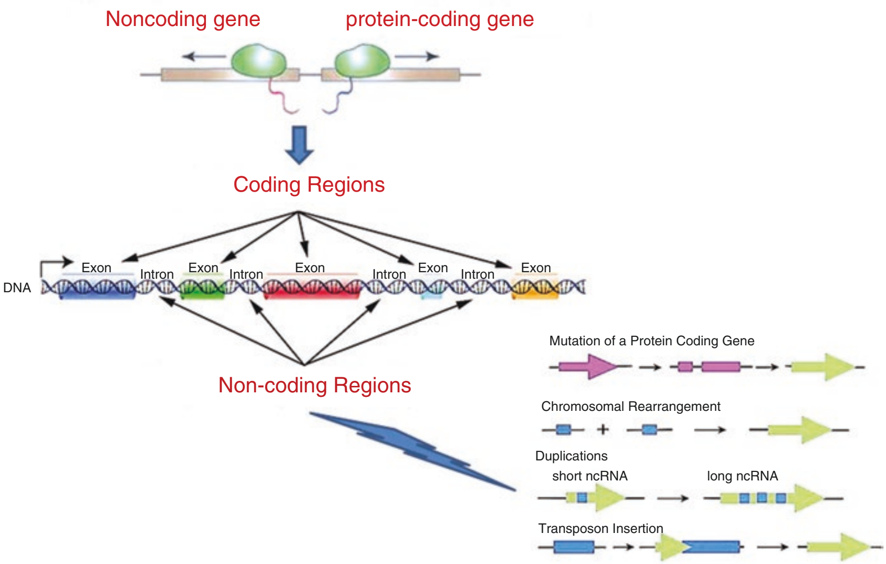


Fig. 5.3 LncRNA genome location

Intronic lncRNA: Intronic lncRNAs are entirely transcribed from introns of protein-coding genes.

Sense lncRNA: Sense lncRNAs are transcribed from the sense strand of protein-coding genes and contain exons from protein-coding genes that overlap with part of protein-coding genes or cover the entire sequence of a protein-coding gene through an intron.

Antisense lncRNA: Antisense lncRNAs are transcribed from the antisense strand of the protein-coding genes that overlap with exonic or intronic regions or cover the entire protein-coding sequence through an intron.

5.5 Bioinformatics Workflows for the Systematic Identification and Annotation of lncRNAs

In order to annotate lncRNAs, a typical workflow (Fig. 5.5) could be applied to the growing number of assembled insect genomes with particular attention on the following three key points. In RNA-Seq protocols, complete transcriptome sequencing (RNA-Seq) represents the method of choice to discover new transcripts and to quantify all RNAs in a variety of organisms and cell types. First, lncRNAs tend to be rare compared to mRNAs and, second, display both spatial and temporal expression patterns, the depth of sequencing, the number of different tissues/cell lines, and time-points needed to be considered in the planning of each experiment. For instance, it has been shown in human and fruit fly that testis tissue shows the highest number of tissue-specific lncRNAs (e.g., 11% of all lncRNAs in *Drosophila*)

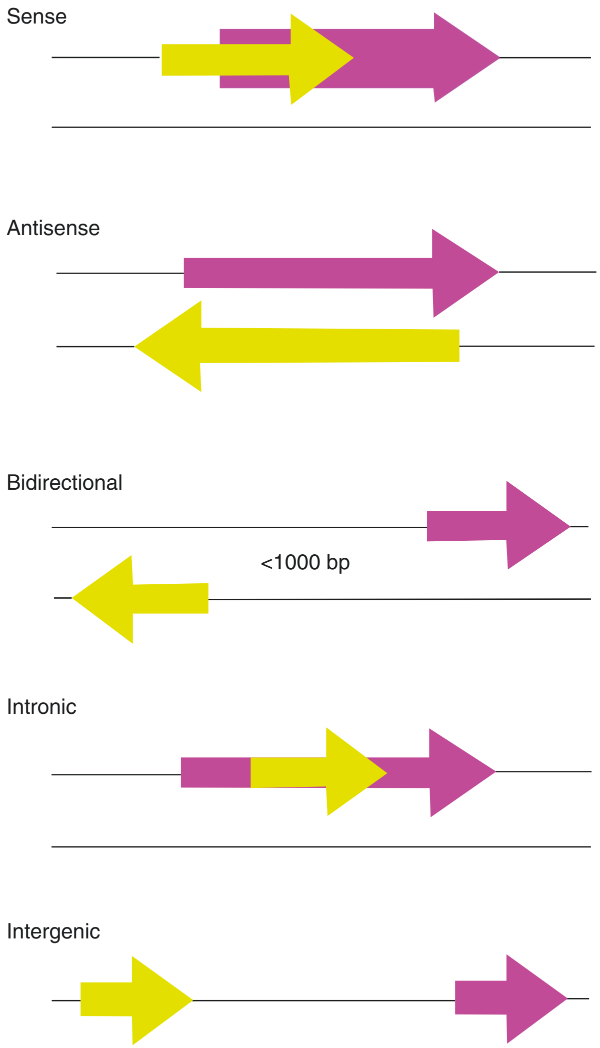


Fig. 5.4 Specific location of lncRNAs

probably reflecting more relaxed chromatin structure (Brown et al. 2014). In addition, many lncRNAs are expressed antisense to protein-coding genes which they often regulate (He et al. 2008; Magistri et al. 2012; Johnsson et al. 2013). It is thus recommended to favor stranded RNA-Seq protocols or directional transcriptome sequencing that keeps track of the strand of origin of the transcript (Ponting et al. 2009), if the purpose of the study includes the identification of antisense lncRNAs. For example, using these stranded protocols, the modENCODE project recently discovered 402 lncRNA loci (21% of all lncRNA loci) located antisense to mRNA transcripts of protein-coding genes in *D. melanogaster* (Brown et al. 2014), while this proportion is slightly lower (15%) in the human genome (Derrien et al. 2012).

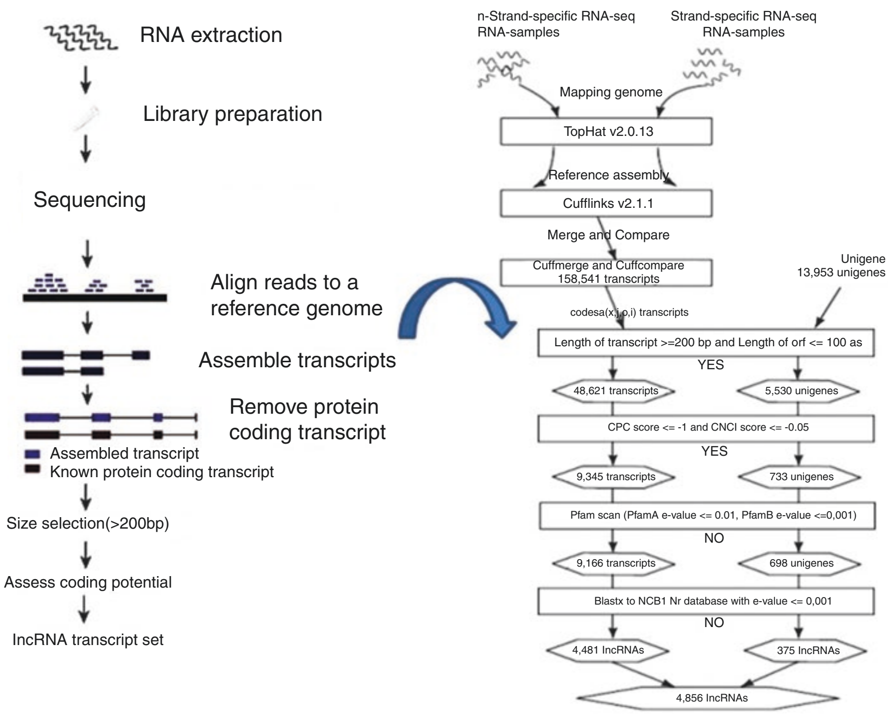


Fig. 5.5 Complete workflow to the identification of lncRNAs in insects

According to our unpublished data of Kumar et al., we identified various lncRNAs by using the following protocol. Briefly, extract the total RNA in RNA-free environment from the targeted tissues of insects, the library should be prepared, and RNA sequencing is used to perform by standard protocols. After alignments of reads, assemble the transcript and remove the protein-coding gene and separate the sequences more than 200 bp and set the data lncRNA transcript (Fig. 5.5). Zhou et al. (2016) elaborated a comprehensive database of noncoding RNAs in the silkworm *B. mori* called as BmncRNAdb and opened a route for researchers engaged in insect molecular biology research particularly long noncoding RNAs. Briefly, genome-wide identification of lncRNAs in the silkworm was analyzed. Two sorts of information from the silkworm were utilized for recognizable proof of the silkworm lncRNAs. The first is the silkworm RNA-Seq information. Forty-one RNA-Seq datasets were distributed by other research that are gathered before the end of 2014, and four RNA-Seq datasets were created by his team (Cheng et al. 2015; Fang et al. 2015; Gong et al. 2014; Kiuchi et al. 2014; Ma et al. 2014; Nie et al. 2014; Nishida et al. 2015; Shao et al. 2012, 2014; Xue et al. 2012; Zemach et al. 2010). All the RNA-Seq information were utilized to recreate the silkworm transcriptome utilizing the product TopHat v2.0.13 and Cufflinks v2.1.1 (Liu et al. 2012; Gong et al. 2014; Ilott and Ponting 2013; Legeai and Derrien 2015; Nam and Bartel 2012; Zhou et al. 2014; Mattick and Rinn 2015; Liao et al. 2014; Zemach

et al. 2010). The second was the silkworm unigenes. The unigene transcripts were combined from expressed sequence tag, and some of the lncRNAs were additionally contained in the unigene transcripts (Zhou et al. 2014). Thus, the transcripts assembled from RNA-Seq data and unigenes were used to identify lncRNAs in his study (Zhou et al. 2016).

5.6 Molecular Cloning of lncRNAs and Gene Expression Work Flow in Insects

Molecular cloning of lncRNAs is a typical process; according to our unpublished report, primers related to specific studies are used to be designed and amplified using PCR. After successful amplification of lncRNA genes, the relative gene expression levels are used to estimate according to the $2^{-\Delta\Delta Ct}$ method (Jacobson et al. 2011). One of the important pIZT-V5/His (Guo et al. 2016) vectors could be adopted to construct recombinant plasmid to transfect the insect cells for gene expression study at the cell level (Fig. 5.6).

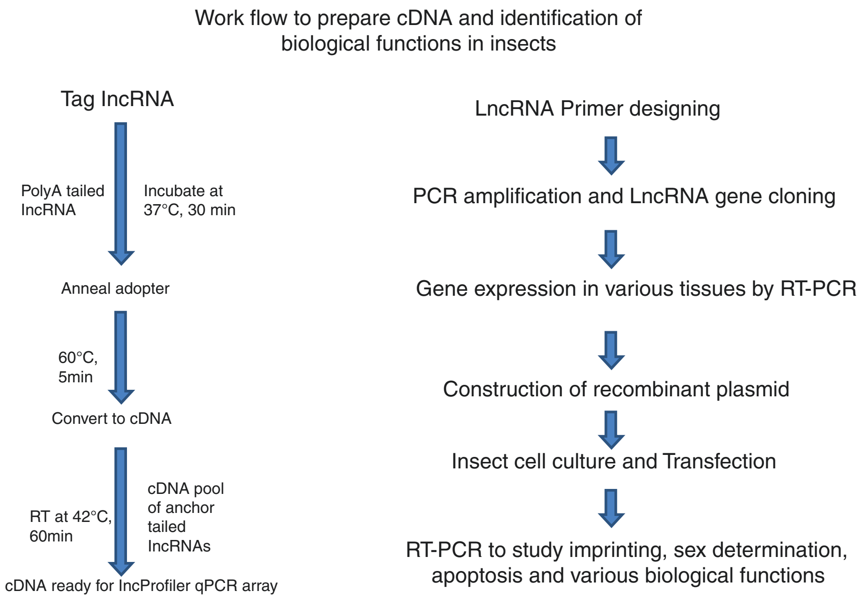


Fig. 5.6 Pipeline of cloning of lncRNAs and gene expression study in insects

5.7 General Mechanism of LncRNAs and Biological Functions in Insects

In general, long noncoding RNAs (lncRNAs) are an imperative class of unavoidable qualities required in an assortment of natural capacities. Here, we examine the rising models of atomic capacities that lncRNAs execute as signalings, decoy, guides, and scaffold. For every original, cases from a few unique natural settings delineate the shared characteristic of the subatomic components, and these unthinking perspectives give valuable clarifications and expectations of organic results. These prime examples of lncRNA capacity might be a helpful structure to consider how lncRNAs get properties as natural flag transducers and allude to their conceivable starting points in development. As new lncRNAs are being found at a fast pace, the atomic components of lncRNAs are probably going to be enhanced and differentiated (Wang and Chang 2011). The mechanism and functions of lncRNA are as follows (Fig. 5.7).

I. Signaling

The transcription of certain lncRNAs is very tissue and temporally specific. Their expression can be in response to certain stimuli, such as cellular stress and temperature. Thus, lncRNAs can serve as molecular signals and can act as markers of functionally significant biological events (Guttman et al. 2009).

II. Decoys

The molecular decoy type of activity takes place when specific lncRNAs are transcribed and then bind to and titrate away protein factors. Decoy lncRNAs can "sponge" protein factors such as transcription factors and chromatin modifiers. This leads to broad changes in the transcriptome of the cell (Guenther et al. 2007; Bernard et al. 2010).

III. Guides

LncRNAs can be molecular guides by localizing particular ribonucleoprotein complexes to specific chromatin targets. This activity can cause changes in gene expression either in cis (on neighboring genes) or in trans (distantly located genes) that cannot be easily predicted by just the lncRNA sequence itself (Bonasio et al. 2010; Lee 2009).

IV. Scaffolds

Assembly of complex protein complexes can be supported by lncRNAs, linking factors together to form new functions. Some lncRNAs possesses different domains that bind distinct protein factors that altogether may impact transcriptional activation or repression (Spitale et al. 2011; Good et al. 2011; Wang and Chang 2011).

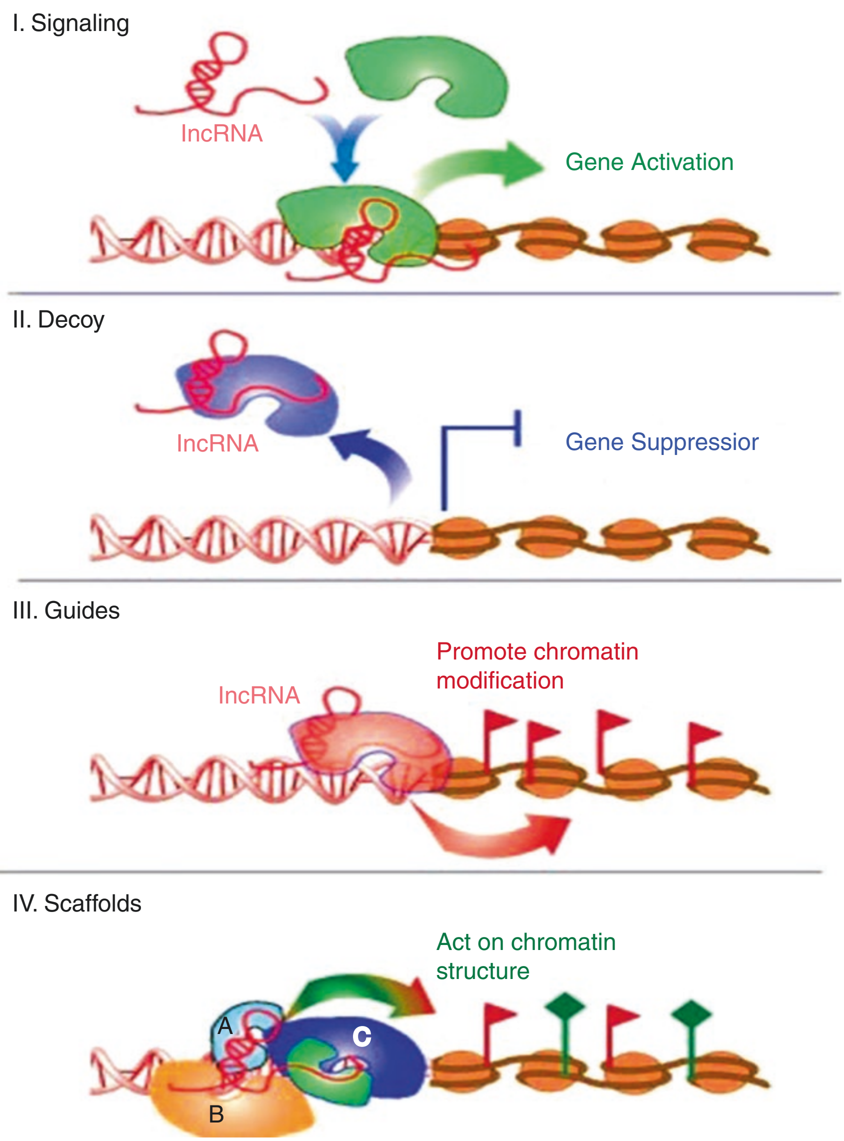


Fig. 5.7 General mechanism of LncRNAs

Conclusion

After discovery of lncRNAs, the diversity of nonprotein-coding genes and their molecular functions has been widely investigated in various important insect research models from tissue to cell level. LncRNAs disclosed the various hidden and key gene functions and also performing significant role in the various diseases of insects. However, its beginning, in the coming future, lncRNAs investigation will uncover the numerous conceals of the other genetically important insects.

References

Adams MD, Celniker SE, Holt RA et al (2000) The genome sequence of *Drosophila melanogaster*. Science 287:2185–2195

Bernard D, Prasanth KV, Tripathi V, Colasse S, Nakamura T et al (2010) A long nuclear-retained non-coding RNA regulates synaptogenesis by modulating gene expression. EMBO J 29:3082–3093

Bonasio R, Shiekhattar R (2004) Regulation of transcription by long noncoding RNAs. Annu Rev Genet 48:433–455

Bonasio R, Tu S, Reinberg D (2010) Molecular signals of epigenetic states. Science 330:612–616

Brown JB, Boley N, Eisman R, May GE, Stoiber MH et al (2014) Diversity and dynamics of the *Drosophila* transcriptome. Nature 512:393–399

Chen Y, Dai H, Chen S, Zhang L, Long M (2011) Highly tissue specific expression of sphinx supports its male courtship related role in *Drosophila melanogaster*. PLoS One 6:e18853

Cheng T, Fu B, Wu Y, Long R, Liu C et al (2015) Transcriptome sequencing and positive selected genes analysis of Bombyx Mandarina. PLoS One 10:e0122837

Deng X, Meller VH (2006) roX RNAs are required for increased expression of X-linked genes in *Drosophila melanogaster* males. Genetics 174:1859–1866

Derrien T, Johnson R, Bussotti G, Tanzer A, Djebali S et al (2012) The GENCODE v7 catalog of human long noncoding RNAs: analysis of their gene structure, evolution, and expression. Genome Res 22:1775–1789

Djebali S, Davis CA, Merkel A, Dobin A, Lassmann T et al (2012) Landscape of transcription in human cells. Nature 488:101–108

Eddy SR (2001) Non-coding RNA genes and the modern RNA world. Nat Rev Genet 2:919–929

Etebari K, Michael J, Furlong S (2015) Intergenic non-coding RNAs in diamondback moth (*Plutella xylostella*) and their expression in insecticide resistant strains. Sci Rep 5:14642

Fabrice L, Thomas D (2015) Identification of long non-coding RNAs in insects genomes. Curr Opin Insect Sci 7:1–8 (Elsevier: 4054001009011)

Fang SM, Hu BL, Zhou QZ, Yu QY, Zhang Z (2015) Comparative analysis of the silk gland transcriptomes between the domestic and wild silkworms. BMC Genomics 16:60

Fatica A, Bozzoni I (2013) Long non-coding RNAs: new players in cell differentiation and development. Nat Rev Genet 15:7–21

Gardini A, Shiekhattar R (2014) The many faces of long noncoding RNAs. FEBS J 282(9):1647–1657. doi:10.1111/febs.13101

Gong L, Chen X, Liu C, Jin F, Hu Q (2014) Gene expression profile of *Bombyx mori* hemocyte under the stress of destruxin a. PLoS One 9:e96170

Good MC, Zalatan JG, Lim WA (2011) Scaffold proteins: hubs for controlling the flow of cellular information. Science 332:680–686

Guenther MG, Levine SS, Boyer LA, Jaenisch R, Young RA (2007) A chromatin landmark and transcription initiation at most promoters in human cells. Cell 130:77–88

Guo R, Guangli C, Yahong L, Renyu X, Dhiraj K et al (2016) Exogenous gene can be integrated into *Nosema bombycis* genome by mediating with a non-transposon vector. Parasitol Res 115:3093–3098

Guttman M, Amit I, Garber M, French C, Lin MF (2009) Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals. Nature 458:223–227

Hao Z, Fan C, Cheng T, Su Y, Wei Q (2015) Genome-wide identification, characterization and evolutionary analysis of long intergenic noncoding RNAs in cucumber. PLoS One 10:e0121800

He Y, Vogelstein B, Velculescu VE, Papadopoulos N, Kinzler KW (2008) The antisense transcriptomes of human cells. Science 322:1855–1857

Holt RA, Subramanian GM, Halpern A et al (2002) The genome sequence of the malaria mosquito *Anopheles gambiae*. Science 298:12–149

Humann FC, Hartfelder K (2011) Representational difference analysis (RDA) reveals differential expression of conserved as well as novel genes during caste-specific development of the honey bee (*Apis mellifera* L.) ovary. Insect Biochem Mol Biol 41:602–612

Ilott NE, Ponting CP (2013) Predicting long non-coding RNAs using RNA sequencing. Methods 63:50–59

Iyer MK, Niknafs YS, Malik R, Singhal U, Sahu A et al (2015) The landscape of long noncoding RNAs in the human transcriptome. Nat Genet 47:199–208

Jacobson A, Baran I, Popovi C, Sorkine O (2011) Bounded biharmonic weights for real-time deformation. ACM Trans Graph 30:78:1–78:8

Jayakodi M, Jung JW, Park D, Ahn YJ, Lee SC et al (2015) Genome-wide characterization of long intergenic non-coding RNAs (lincRNAs) provides new insight into viral diseases in honey bees Apis Cerana and Apis Mellifera. BMC Genomics 16:680

Jenkins AM, Waterhouse RM, Muskavitch MA (2015) Long non-coding RNA discovery across the genus anopheles reveals conserved secondary structures within and beyond the Gambiae complex. BMC Genomics 16:337

Jia L, Dayan Z, Zhonghuai X, Ningjia H (2015) Nonfunctional ingestion of plant miRNAs in silkworm revealed by digital droplet PCR and transcriptome analysis. Sci Rep 5:12290

Johnsson P, Ackley A, Vidarsdottir L, Lui WO, Corcoran M et al (2013) A pseudogene long-noncoding-RNA network regulates PTEN transcription and translation in human cells. Nat Struct Mol Biol 20:440–446

Kapranov P, Laurent G (2012) Dark matter RNA: existence, function and controversy. Front Genet 3:60

Kapranov P, Cheng J, Dike S, Nix DA, Duttagupta R et al (2007) RNA maps reveal new RNA classes and a possible function for pervasive transcription. Science 316:1484–1488

Kawaoka S, Kadota K, Arai Y, Suzuki Y, Fujii T et al (2011) The silkworm W chromosome is a source of female-enriched piRNAs. RNA 17:2144–2151

Kiuchi T, Koga H, Kawamoto M, Shoji K, Sakai H et al (2014) A single female-specific piRNA is the primary determiner of sex in the silkworm. Nature 509(7502):633–636

Kiya T, Kunieda T, Kubo T (2008a) Inducible- and constitutive-type transcript variants of kakusei, a novel non-coding immediate early gene, in the honeybee brain. Insect Mol Biol 17:531–536

Kiya T, Kunieda T, Kubo T (2008b) Inducible and constitutive-type transcript variants of kakusei, a novel noncoding immediate early gene, in the honeybee brain. Insect Mol Bio 17:531–536

Kiya T, Ugajin A, Kunieda T, Kubo T (2012) Identification of kakusei, a nuclear non-coding RNA. Molecular Sci 13:15496–15509

Kowalczyk MS, Higgs DR, Gingeras TR (2012) Molecular biology: RNA discrimination. Nature 482:310–311

Kutter C, Watt S, Stefflova K, Wilson MD, Goncalves A et al (2012) Rapid turnover of long noncoding RNAs and the evolution of gene expression. PLoS Genet 8:e1002841

Lakhotia SC (2011) Forty years of the 93D puff of *Drosophila melanogaster*. J Biosci 36:399–423

Lakhotia SC, Mallik M, Singh AK, Ray M (2012) The large noncoding hsrv-n transcripts are essential for thermotolerance and remobilization of hnRNPs, HP1 and RNA polymerase II during recovery from heat shock in *Drosophila*. Chromosoma 121:49–70

Lee JT (2009) Lessons from X-chromosome inactivation: long ncRNA as guides and tethers to the epigenome. Genes Dev 23:1831–1842

Legeai F, Derrien T (2015) Identification of long non-coding RNAs in insects genomes. Curr Opin Insect Sci 7:37–44
Li M, Wen S, Guo X, Bai B, Gong Z et al (2012) The novel long non-coding RNA CRG regulates *Drosophila* locomotor behavior. Nucleic Acids Res 40:11714–11727
Li L, Eichten SR, Shimizu R, Petsch K, Yeh CT et al (2014) Genome-wide discovery and characterization of maize long non-coding RNAs. Genome Bio 5:40
Liao Q, Shen J, Liu J, Sun X, Zhao G et al (2014) Genome-wide identification and functional annotation of plasmodium falciparum long noncoding RNAs from RNA-seq data. Parasitol Res 113:1269–1281
Lim LP, Glasner ME, Yekta S et al (2003a) Vertebrate microRNA genes. Science 299:1540
Lim LP, Lau NC, Weinstein EG et al (2003b) The microRNAs of *Caenorhabditis elegans*. Genes Dev 17:991–1008
Lipshitz HD, Peattie DA, Hogness DS (1987) Novel transcripts from the Ultrabithorax domain of the bithorax complex. Genes Dev 1:307–322
Liu J, Jung C, Xu J, Wang H, Deng S et al (2012) Genome-wide analysis uncovers regulation of long intergenic noncoding RNAs in Arabidopsis. Plant Cell 24:4333–4345
Ma L, Ma Q, Li X, Cheng L, Li K (2014) Transcriptomic analysis of differentially expressed genes in the Ras1(CA)-overexpressed and wildtype posterior silk glands. BMC Genomics 15:182
Magistri M, Faghihi MA, St Laurent G, Wahlestedt C (2012) Regulation of chromatin structure by long noncoding RNAs: focus on natural antisense transcripts. Trends Genet 28:389–396
Mattick JS, Rinn JL (2015) Discovery and annotation of long noncoding RNAs. Nat Struct Mol Bio 22:5–7
Mercer TR, Dinger ME, Mattick JS (2009) Long non-coding RNAs: insights into functions. Nat Rev Genetics 10:155–159
Nam JW, Bartel DP (2012) Long noncoding RNAs in *C. elegans*. Genome Res 22:2529–2540
Necsulea A, Soumillon M, Warnefors M, Liechti A, Daish T et al (2014) The evolution of lncRNA repertoires and expression patterns in tetrapods. Nature 505:635–640
Nie H, Liu C, Cheng T, Li Q, Wu Y et al (2014) Transcriptome analysis of integument differentially expressed genes in the pigment mutant (quail) during molting of silkworm, *Bombyx mori*. PLoS One 9:e94185
Nishida KM, Iwasaki YW, Murota Y, Nagao A, Mannen T et al (2015) Respective functions of two distinct Siwi complexes assembled during PIWI-interacting RNA biogenesis in Bombyx germ cells. Cell Rep 10:193–203
Padron A, Molina-Cruz A, Quinones M, Ribeiro JM, Ramphul U et al (2014) In depth annotation of the *Anopheles gambiae* Mosquito midgut transcriptome. BMC Genomics 15:636
Pan MH, Cai XJ, Liu M (2010) Establishment and characterization of an ovaria cell line of the silkworm, *Bombyx mori*. Tissue Cell 42:42–46
Pauli A, Valen E, Lin MF, Garber M, Vastenhouw NL et al (2012) Systematic identification of long noncoding RNAs expressed during zebrafish embryogenesis. Genome Res 22:577–591
Pease B, Borges AC, Bender W (2013) Noncoding RNAs of the ultrabithorax domain of the *Drosophila* bithorax complex. Genetics 195:1253–1264
Ponting CP, Oliver PL, Reik W (2009) Evolution and functions of long noncoding RNAs. Cell 139:629–641
Sawata M, Daisuke Y, Takeuchi H, Kamikouchi A, Kazuaki O, Kubo T (2002) Identification and punctate nuclear localization of a novel noncoding identification and punctate nuclear localization of a novel noncoding RNA, Ks-1, from the honeybee brain. RNA 8(6):772–785
Sawata M, Takeuchi H, Kubo T (2004) Identification and analysis of the minimal promoter activity of a novel noncoding nuclear RNA gene, AncR-1, from the honeybee (*Apis mellifera* L.) RNA 10(7):1047–1058. doi:10.1261/rna.5231504.use
Shao W, Zhao QY, Wang XY, Xu XY, Tang Q et al (2012) Alternative splicing and trans-splicing events revealed by analysis of the *Bombyx mori* transcriptome. RNA 18(7):1395–1407
Shi X, Ming S, Hongbing L, Yanwen Y, Rong K (2015) A critical role for the long non-coding RNA GAS5 in proliferation and apoptosis in non-small-cell lung cancer. Mol Carcino 54(Suppl 1):E1–E12

Shoji K, Hara K, Kawamoto M, Kiuchi T, Kawaoka S et al (2014) Silkworm HP1a transcriptionally enhances highly expressed euchromatic genes via association with their transcription start sites. Nucleic Acids Res 42:11462–11471

Smith ER, Allis CD, Lucchesi JC (2001) Linking global histone acetylation to the transcription enhancement of X-chromosomal genes in *Drosophila* males. J Biol Chem 276:31483–31486

Soshnev AA, Li X, Wehling MD, Geyer PK (2008) Context differences reveal insulator and activator functions of a Su(Hw) binding region. PLoS Genet 4:e1000159

Soshnev AA, Ishimoto H, McAllister BF, Li X, Wehling MD (2011) A conserved long noncoding RNA affects sleep behavior in *Drosophila*. Genetics 189:455–468

Spitale RC, Tsai MC, Chang HY (2011) RNA templating the epigenome: long noncoding RNAs as molecular scaffolds. Epigenetics 6:539–543

Sun J, Lin Y, Wu J (2013) Long non-coding RNA expression profiling of mouse testis during postnatal development. PLoS One 8:e75750

Tadano H, Yamazaki Y, Takeuchi H, Kubo T (2009) Age and division-of-labour-dependent differential expression of a novel non-coding RNA, Nb-1, in the brain of worker honeybees, *Apis mellifera* L. Insect Mol Biol 18:715–726

Taguchi S, Iwami M, Kiya T (2011) Identification and characterization of a novel nuclear noncoding RNA, Fben-1, which is preferentially expressed in the higher brain center of the female silkworm moth, *Bombyx mori*. Neurosci Lett 496:176–180

Tatsushi I, Masayuki M (2004) Role of Bcl-2 family members in invertebrates, Biochim. Biophys Acta 1644:73–81

Tran UM, Rajarajacholan U, Soh J, Kim T-S (2015) LincRNA-p21 acts as a mediator of ING1b-induced apoptosis. Cell Death Dis 6:e1668. doi:10.1038/cddis

Ulitsky I, Bartel DP (2013) LincRNAs: genomics, evolution, and mechanisms. Cell 154:26–46

Wahlestedt C (2013) Targeting long non-coding RNA to therapeutically upregulate gene expression. Nat Rev Drug Dis 12:433–446

Wang KC, Chang HY (2011) Molecular mechanisms of long noncoding RNAs. Mol Cell 43:904–914

Wang Z, Gerstein M, Snyder M (2009) RNA-Seq: a revolutionary tool for transcriptomics. Nat Rev Genet 10:57–63

Wang M, Yuan D, Tu L, Gao W, He Y et al (2015) Long noncoding RNAs and their proposed functions in fibre development of cotton (Gossypium spp.) New Phytol 207:1181–1197

Wu W, Zhang S, Li X, Xue M, Cao S et al (2013) Ets-2 regulates cell apoptosis via the Akt pathway, through the regulation of urothelial cancer associated 1, a long non-coding RNA, in bladder cancer cells. PLoS One 8:e73920

Wu Y, Cheng T, Liu C, Liu D, Zhang Q et al (2016) Systematic identification and characterization of long non-coding RNAs in the silkworm, *Bombyx mori*. PLoS One 11:e0147147

Xue J, Qiao N, Zhang W, Cheng RL, Zhang XQ et al (2012) Dynamic interactions between *Bombyx mori* nucleopolyhedrovirus and its host cells revealed by transcriptome analysis. J Virol 86:7345–7359

Young RS, Marques AC, Tibbit C, Haerty W, Bassett AR et al (2012) Identification and properties of 1,119 candidate lincRNA loci in the *Drosophila melanogaster* genome. Genome Bio Evol 4:427–442

Zemach A, McDaniel IE, Silva P, Zilberman D (2010) Genome-wide evolutionary analysis of eukaryotic DNA methylation. Science 328:916–919

Zhang YC, Liao JY, Li ZY, Yu Y, Zhang JP et al (2014) Genome-wide screening and functional analysis identify a large number of long noncoding RNAs involved in the sexual reproduction of rice. Genome Bio 15:512

Zhou ZY, Li AM, Adeola AC, Liu YH, Irwin DM et al (2014) Genome-wide identification of long intergenic noncoding RNA genes and their potential association with domestication in pigs. Genome Biol Evol 6:1387–1392

Zhou QZ, Bindan Z, Quan-You Y, Ze Z (2016) BmncRNAdb: a comprehensive database of non-coding RNAs in the silkworm, *Bombyx mori*. BMC Bioinformatics 17:370

Pathogen-Driven Proteomic Changes in Hemolymph of Nuclear Polyhedrosis Virus-Infected Silkworm *Bombyx mori* L.

6

M. Sayed Iqbal Ahamad, Neetha N. Kari, and Shyam Kumar Vootla

Abstract

Viral infections are distinct in the capacity of the viruses to overtake the host's protein synthesis machinery and regulate it for the viral replication. Interaction and infection process of BmNPV in its host *Bombyx mori* is an important step to understand host-pathogen interaction studies. In present research work, we have screened the BmNPV isolate from the grasserie-infected silkworms from rearing fields of Central Karnataka, India. Isolation, purification, and characterization of the BmNPV virus were done by sucrose density gradient centrifugation, scanning electron microscopy, and SDS-PAGE of the occlusion bodies (OBs). Comparative proteomic analysis revealed drastic up- and downregulation of several proteins in control and infected silkworms. The role of various proteins in comparison with reported proteins responsible for disease infection was elucidated.

6.1 Introduction

6.1.1 Introduction to Baculoviruses

Baculoviruses are a group of insect-specific viruses belonging to the family *Baculoviridae*. They are ubiquitous in the environment and an important contributor

M. Sayed Iqbal Ahamad • S.K. Vootla (✉)
Department of Biotechnology and Microbiology, Karnatak University, 580 003 Dharwad, India
e-mail: iqbalahmed83@gmail.com; vootlashyam@kud.ac.in

N.N. Kari
Department of Zoology, Karnatak University, Dharwad, India
e-mail: neetakari@gmail.com

D. Kumar, C. Gong (eds.), *Trends in Insect Molecular Biology and Biotechnology*, https://doi.org/10.1007/978-3-319-61343-7_6

to insect population regulation. Host insects are considered as model system to study the viral pathogenesis; the interactions between the host and viruses during pathogenesis lead to several physiological changes including protein regulation. Studies related to host-pathogen interactions are essential in exploring insect viruses as biocontrol agents for insect pest control.

Baculoviruses comprise a diverse group of anthropocentric, circular double-stranded DNA viruses with the genome of 80–180 kb in size, which are packaged within a rod-shaped capsid, enclosed in a lipid envelope. Baculoviruses are reported in more than 600 insect species of the orders Lepidoptera, Hymenoptera, Diptera, Coleoptera, Neuroptera, Trichoptera, and Thysanura, including Decapoda of Crustacea (Murphy et al. 1995).

The life cycle of baculoviruses is biphasic with two different phenotypes during infective stage, which includes occlusion-derived virions (ODVs) and budded virions (BVs). Morphologically ODVs and BVs are different but genetically same. The specific feature of NPV infection is the production of crystalline proteinaceous structures called occlusion bodies (OBs) or polyinclusion bodies (PIBs), in which several ODVs are embedded and are protected from adverse environmental factors such as UV light, temperature, desiccation, proteases, and nucleases activity, whereas budded viruses are free from proteinaceous matrix and are involved in cell to cell vertical transmission during the infection. Morphologically there are two subgroups in NPVs, viz., the single nucleocapsid NPV (e.g., BmNPV), in which only one nucleocapsid is present per envelope, and the multinucleocapsid NPVs (e.g., AcMNPV) in which several nucleocapsids are packaged per envelope. As the viruses do not possess independent molecular machinery for replication, they replicate in the nuclei of host cells.

6.1.2 Replication of Baculoviruses

The infectivity of baculoviruses to the specific host insect is of two modes, viz., horizontal transmission and vertical transmission. Baculoviruses replicate by ODVs embedded in OBs that are produced in the final stage of the replication cycle and are released upon the death and disintegration of the insect. The alkaline microenvironment of insect midgut with pH 8–11 dissolves the OBs, within few seconds ODVs are released. The released virions infect midgut epithelial cells through peritrophic membrane (PM). The metalloproteinases and enhancins are concentrated in occlusion bodies, digest mucin on the midgut, and allow virus to access the epithelial cell surface (Wang and Granados 1997). Then virion envelope fuses with the microvillar membrane, allowing the nucleocapsids to enter the microvilli; these cells produce the BVs. BVs are responsible for systemic vertical infection to the surrounding healthy cells. They enter other cell through receptor-mediated endocytosis. After the penetration of the plasma membrane, the nucleocapsid uncoats and releases viral DNA in the nucleus to initiate viral DNA replication. Finally, along with hemolymph, BVs circulate throughout the body and infect other tissues of the host.

After 12 h of postinfection, mature ODVs embedded in the envelope get packaged into the OBs due to the overexpression of polyhedrin (polh), a structural protein. At the last stage of infection, larvae stop feeding and exhibit melanization of cuticle, intersegmental swelling, flaccid musculature, and wandering movements; ultimately death and disintegration of larvae takes place. Larval disintegration and contamination of fecal matter of infected larvae by OBs lead to horizontal transmission of the disease to other healthy larvae.

6.1.3 Structural Proteins of Baculoviruses

Immobilization of virus within the crystalline protein lattice of the occlusion body allows virions to remain viable indefinitely from extremes of heat and UV radiations.

6.1.3.1 Structural Proteins of Occlusion Bodies (OBs)

Polyhedrin (BmNPV orf 1) Polyhedrin is a major structural component of OBs. It is composed of about 250 amino acids (30 kDa) and is one of the most conserved baculovirus protein. It forms a crystalline cubic lattice to protect embedded virions. The 30 kDa subunits form trimers that are arranged into dodecamers via disulfide bonds. This structure interlocks with another dodecamer to form the cubic-shaped unit cell of the crystal. Hydrophobic and salt bridge interactions between the cubes form the linkages at the crystal interfaces that are disrupted by the alkaline pH of the insect midgut; the digestion of polyhedrin enables release of virions.

The Calyx/Polyhedron Envelope (BmNPV orf 108) The calyx/polyhedron envelope (PE) is an electron-dense structure that forms a smooth, seamless surface that surrounds OBs. The function of the calyx/PE is to seal the surface of OBs and to enhance their stability. Homologs of the polyhedron envelope (PE) protein (Ac131) are found in the genomes of all lepidopteran NPVs. It is likely that when OBs are ingested by susceptible insects, they are dismantled by a combination of the alkaline pH of the insect midgut and proteinases. This combination contributes to the disruption of the polyhedron and polyhedron envelope to facilitate the release of virions.

6.1.3.2 Associated Proteins of OBs

Ac68 Homologs of Ac68 appear to be present in all baculoviruses. It may be involved in polyhedron morphogenesis (Xu 2008).

P10 (BmNPV orf 114) Although p10 does not appear to be a major occlusion body protein, it colonizes with the PE protein and appears to be required for the formation of the polyhedron envelope (Gross et al. 1994).

Viral-Enhancing Factor (Enhancins) Enhancins are a class of metalloproteinases that are encoded by a few lepidopteran NPVs. The viral-enhancing factor is a 104 kDa

protein that forms about 5% of the mass of OBs. Enhancin is thought to facilitate baculovirus infection by digesting the peritrophic membrane (Lepore et al. 1996).

6.1.3.3 Structural Proteins of ODVs

Envelope Proteins of ODVs

BV/ODV-E26 (Ac16) This protein is known to delay BV production. It has also shown to interact with both IE-1 and IE-0 proteins and may be involved in the regulation of other gene products (Nie et al. 2009).

ODV-E66 (BmNPV orf 37) ODV-E66 is a component of ODV envelopes. Evidence suggests that BmNPV orf 37 encodes an enzyme hyaluronan lyase with the molecular weight of 60 kDa. It is capable of digesting hyaluronan, a polysaccharide that is a major component of the extracellular matrix which provides structural support for the cells (Vigdorovich et al. 2007).

ODV-E25 (BmNPV orf 77) It is localized to ODV envelopes of 25 kDa molecular weight (Russell and Rohrmann 1993).

ODV-EC43 (Ac109) Homologs of Ac109 are present in all baculovirus genomes. Although deletion of Ac109 does not appear to affect DNA replication or the appearance of BV, the virions become noninfectious (Fang et al. 2007).

ODV-E18 (BmNPV orf 119/120) It is a protein of 18 kDa in the ODV envelope fraction. Deletion of the ODV-E18 gene results in single-cell infections that produce OBs and therefore appears to be essential for BV production (McCarthy and Theilmann 2008).

ODV-E56, PIF-5 (BmNPV orf 124) ODV-E56 localizes to the envelopes of occluded virions. Homologs of ODV-E56 are present in all the baculovirus genomes. ODV-E56 has been shown to be a per os infectivity factor required for midgut infection (Braunagel et al. 1996).

Additional Envelope Proteins of ODVs (Per os Infectivity Factors)

P74-Pif-0 (BmNPV orf 115), Pif-1 (BmNPV orf 97), Pif-2 (BmNPV orf 13), Pif-3 (BmNPV orf 95), Pif-4 (BmNPV orf 79), and ODV-E56, Pif-5 (BmNPV orf 124) These per os infectivity factors are required for infection of insects. PIF-1, PIF-2, and p74 mediate specific binding of occlusion-derived virus to midgut cells.

Ac145 and Ac150 These two genes encode small proteins ~9 and 11 kDa, respectively, that are related to one another. Ac145 and Ac150 were found to be associated with both BV and ODV. Evidence suggested that the mutant had a reduction in its ability to establish primary infections in midgut cells (Zhang 2005).

6.1.3.4 Structural Proteins of Nucleocapsids

P6.9 (BmNPV orf 84) P6.9 is a DNA-binding protein of BV. It is a small arginine/serine/threonine rich protein. The homologs of P6.9 appear to be encoded by all baculovirus genomes. It localizes in the nuclear matrix during infection. Once viral DNA has been delivered to the nucleus, P6.9 is phosphorylated resulting in DNA binding (Wang 2010).

VLF-1 (Ac77) It influences the hyperexpression of the very late genes. VLF-1 appears to be a structural protein present in both BVs and ODVs and is clearly required for the production of nucleocapsids (Yang and Miller 1998).

VP39 (BmNPV orf 72) VP39 is thought to be the major capsid protein with 39 kDa.

GP41 Tegument Protein (BmNPV orf 66) GP41 homologs are present in all baculovirus genomes; it is 41 kDa protein required for the release of nucleocapsids from the nucleus.

Ac98 (38K) Ac98 is encoded by all baculoviruses and is associated with both BV and ODV nucleocapsids. When deleted, tubelike structures devoid of DNA are produced (Wu 2006).

Ac141 (exon0) Ac141 is conserved in all lepidopteran baculoviruses and is associated with both BV and ODV nucleocapsids. It appears to be required for the efficient transport of nucleocapsids from nuclei through the cytoplasm (Fang et al. 2007).

p49 (Ac142) p49 is associated with both BV and ODV virions. Deletion of Ac142 affects nucleocapsid formation but does not affect DNA synthesis (Vanarsdall et al. 2007).

Ac144 Ac144 encodes a virion-associated protein; it is a multifunctional cyclin and may be involved in regulating the cell cycle during virus infection (Belyavskyi et al. 1998).

6.1.3.5 Other Structural Proteins

PP78/83 (Ac9) It is a phosphorylated protein that is located at one end of nucleocapsids. It is a Wiskott-Aldrich syndrome protein (WASP)-like protein, involved in nuclear actin assembly (Russell et al. 1997).

BV/ODV-C42 (Ac101) Ac101 encodes a capsid-associated protein. The deletion study of Ac101 affects nucleocapsid formation but does not affect DNA synthesis.

6.1.4 Silkworm Hemolymph Proteins

The silkworm *Bombyx mori* has open circulatory system filled with hemolymph. The hemolymph plays a very important role in transporting nutrients, oxygen, enzymes, and hormones. It is a very good reservoir of nutrition and energy. The larval stage of silkworm is the feeding stage, wherein it derives nutrition for its life processes. This stage is a very crucial period for enhanced metabolic activities and synthesis of immune proteins during infections. The fifth instar is the intermediate phase to produce silk proteins and metamorphosis from larva to pupa. The activity of transferase, phosphatase, and other metabolic enzymes for carbohydrate and lipid metabolism drastically changes at this stage.

Recent proteomic studies on fifth instar silkworm hemolymph proteins revealed variations in 30 and 80 kDa proteins. These two proteins are predicted to be the storage proteins. In this feeding and energy accumulating stage, essential enzymes for metabolism like aldose reductase, glyoxylate reductase, hydroxypyruvate isomerase, and aminoacylase are upregulated. For the metamorphosis from larva to pupa, proteins like beta-N-acetyl glucose aminidase, chitinolytic enzymes, juvenile hormone-binding protein, and imaginal disk growth factor overexpressed. Proteins for the biosynthesis of silk and metamorphosis dehydrogenase, hypothetical proteins, alcohol dehydrogenase II (fragment), transcriptional regulator, and HAD-type hydrolase/phosphatase related to fatty acid biosynthesis are identified. The upregulation of immune proteins like hemolin, prophenoloxidase, serine proteases, paralytic peptide-binding protein, and trypsin inhibitors is also reported (Li et al. 2006; Hou et al. 2010).

6.1.5 Host-Pathogen Interactions of BmNPV and Silkworms

The molecular mechanism of insect resistance to viral infection, recognition of infected cells, and metabolic alterations in the cell or physiological adjustments in the infected cells is poorly understood. Post viral infection, metabolic changes of insect plays an important role in the interaction between the host and pathogen as a part of survival strategy by the presence of physical barriers like cuticle and peritrophic matrix, epithelial barriers, and protease cascades leading to coagulation and melanization and also the production of certain metabolic end products (Lehane et al. 2004). Some of the important aspects of host-pathogen interactions during BmNPV infection are discussed below.

6.1.5.1 Entry into Nuclei

After cell entry, NPV nucleocapsids are transported to the nuclear membrane by actin polymerization (Ohkawa et al. 2010). Nucleocapsids transport through nuclear pores, dock with them, and form nuclear pore complex. In the nucleus, transcriptional cascade initiates and replicates nucleocapsids. After nucleocapsids are replicated in the nucleus of midgut epithelial cells, they need to exit the cell to spread the

infection and bud out from the nucleus with the envelope from the nuclear membrane (Granados and Lawler 1981).

6.1.5.2 Transiting the Basal Lamina

The tracheal projections of basal lamina help viruses to access the tracheal system and allow the virions to move fast and establish systemic infection (Engelhard 1994). Recently, it has been reported that a viral encoded ortholog of fibroblast growth factor (FGF) may be involved in the movement of the virus across the basal lamina (Rohrmann 2011).

6.1.5.3 The Virogenic Stroma

It is a characteristic electron-dense, chromatin-like structure surrounded by less dense spaces found near the center of nuclei of NPV-infected cells. It is presumed to be a molecular scaffold produced for coordinated transcription and replication of viral DNA and the subsequent packaging of DNA and assembly of nucleocapsids.

6.1.5.4 Viral Proteins Involved in the Infection Cycle

The replication cycle includes some viral encoded proteins that have been identified to facilitate replication process.

6.1.5.5 PP78/PP83 and P10

Post baculoviral infection, actin moves into nuclei and subsequently is polymerized from G-actin into F-actin. G-actin is a globular monomeric form of actin and polymerizes into filamentous F-actin. A cellular complex of up to seven proteins is called the Arp2/Arp3 complex. This complex is involved in nucleating the formation of F-actin filaments. Activators are required for this process, and they bind both monomeric G-actin and the Arp2/Arp3 complex. One category of such activators is called Wiskott-Aldrich syndrome protein (WASP), and an ortholog of WASP (PP78/PP83) (AcMNPV orf 9) is encoded by all lepidopteran NPV genomes. The actin polymerization is required for the coordination of nucleocapsid development including the proper association of ODV with envelopes. The Pl0 protein of the virus is responsible for the lysis of infected cells and the disintegration of the nucleus (Goley 2006).

6.1.5.6 Ecdysteroid UDP-Glucosyltransferase (EGT) (Ac15)

The viral protein that can affect the course of infection is an enzyme ecdysteroid UDP-glucosyltransferase (Wang 1995). The function of the viral EGT is to block molting and pupation in infected larvae by catalyzing the transfer of glucose from UDP-glucose to ecdysteroids; viral infection inactivates insect molting hormones. Molting can cause severe physiological stress on infected insects, and many do not survive this transition. To fulfill complete infection, molting should be delayed. It also prolongs the feeding stage of infected larvae, thereby allowing the virus to replicate over a longer period of time in larger larvae, resulting in a higher yield of virus.

A remarkable feature of NPV infection is that in some instances the insects can grow and continue feeding right until pupation. They appear healthy yet when examined are heavily infected with high concentrations of occlusion bodies in their cells and hemolymph. A feature of the final stage of baculovirus replication that takes place after DNA replication has occurred is the hyperexpression of very late genes resulting in the production of high levels of polyhedrin and p10. Polyhedrin accumulates in nuclei and at some point crystallizes into a lattice that surrounds virions.

The dispersal of virus takes place by the OBs from the infected larvae. The infected insects migrate to a higher elevation on the branch of the tree. This facilitates easy dispersal of the OBs. A gene (Ac1) encoding an RNA processing enzyme (RNA 5′-triphosphatase) has implicated characteristic terminal movement of infected insects. Late in infection after the wandering stage, the insects undergo disintegration or liquefaction at later stage.

6.1.5.7 Enzymes Facilitating Disintegration of Host Insect

Chitinase (Ac126) and Cathepsin (Ac127) Some baculoviruses express chitinase of 59.8 kDa, which facilitate the disintegration of infected larvae. They play key role in virus dispersal. The insect exoskeleton is composed of chitin. In conjunction with another enzyme, a viral proteinase (cathepsin, Ac127), chitinase participates in the liquefaction of insects at late infection.

Recently many proteins are identified from the host and pathogen which play key role during infection and spreading the infection. In case of *Lymantria dispar* multicapsid nuclear polyhedrosis virus (LdMNPV) which carries a gene host range factor 1 (hrf-1) codes for 25.7 kDa protein, it inhibits shutdown of protein synthesis in infected cells and supports for viral replication (Du and Thiem 1997).

The major route of baculovirus infection is through the food. To encounter this mode of infection, antiviral red fluorescent proteins are synthesized in gut juice of BmNPV-infected silkworms. Some recent studies on lipases show antiviral activity against BmNPV. The Bmlipase-1, a lipase purified from the digestive juice of *Bombyx mori* larvae, proved to have a strong antiviral activity against BmNPV (Ponnuvel et al. 2003).

In addition to these antiviral proteins, serine proteases of host insect regulate several defense responses like hemolymph coagulation, antimicrobial peptide synthesis, and melanization of pathogen surfaces. Serine protease is identified in the digestive juice of silkworm larvae with strong antiviral activity against BmNPV (Nakazawa et al. 2004). Some lepidopteron larvae resist baculovirus infection by selective apoptosis of infected cells from the midgut epithelial cells and by sloughing off infected cells from the midgut. The viral load has direct influence on lepidopteron insect immune responses and physiological countermeasures against infections (Terenius 2004).

Many studies on baculovirus antiviral defense mechanism were elucidated on hemolin, an insect immunoglobulin (Ig), present in the midgut and hemolymph of host insects. Double-stranded RNA-dependent protein kinase (PKR) also reported for viral inhibition as interferons, found in uninfected cells (Clemens and Elia 1997). The

increased levels of PKR activity are known to induce the apoptosis in response to viral infections. But the baculovirus has developed countermeasures to combat the antiviral defense mechanism of the host by synthesizing antiapoptotic proteins like p35 and inhibitors of apoptosis (IAP), so as to prevent cell death induced by the insect cell apoptotic mechanism (Prudhomme and Couble 2002). The feces of silkworm larvae also possess an antiviral substance L4-1 that shows antiviral activity by damaging viral proteins by producing reactive oxygen species (Lim et al. 2002).

6.2 BmNPV Source and Isolation

Grasserie-infected silkworms were collected from the sericulture fields of Dharwad and Shiggaon of Central Karnataka, India. Hemolymph was collected by puncturing larval proleg by a sterilized scissor. Outflowing hemolymph was immediately transferred into a sterile and prechilled Eppendorf Tube containing a few crystals of phenylthiourea (PTU) and immediately stored at −20 °C.

6.2.1 Purification of Occlusion Bodies (OBs) by Sucrose Density Gradient Centrifugation

The collected hemolymph was centrifuged at 8000 rpm for 10 min at room temperature. The pelleted OBs were further washed in Milli-Q water by centrifugation at 5000 rpm for 10 min. The washing process was repeated and then OBs were collected as a white pellet.

The partially purified OBs were mixed in 1 mL of 0.2% Triton X-100. This solution was layered on 35–65% sucrose gradients and centrifuged at 20,000 rpm for 1 h at 4 °C in SW55Ti rotor using the Beckman Coulter Optima LE-80K Ultracentrifuge.

The purified OBs were suspended in 0.5% SDS and observed in Olympus SZX16 stereo zoom microscope at 20× and 40× magnifications.

6.2.2 Isolation and Purification of Occlusion-Derived Virions (ODVs) from Occlusion Bodies (OBs)

The ODVs were isolated and purified as described by Braunagel and Summers (2007); accordingly, the OB suspension was incubated in polyhedra lysis buffer (0.1 M Na_2CO_3; 0.166 M NaCl; 0.01 M EDTA, pH 10.5) at 37 °C for 2 h, and the suspension was neutralized by using 0.5 M Tris-HCl (pH 7.5). The alkali-treated OB suspension was layered onto the 35–65% (w/v) sucrose gradients and centrifuged at 28,000 rpm for 1 h. The purified band of ODVs was collected and stored at −20 °C.

The purity of the OBs was confirmed by microscopic observation in Olympus SZX16 stereo zoom microscope at 40× magnification. The density of the OB suspension was adjusted to 5×10^7 OBs/mL using Blankenburg Neubauer chamber.

6.2.3 Scanning Electron Microscopy of Occlusion Bodies (OBs) of BmNPV

The purified OBs were smeared on stubs on previously covered carbon tape and allowed to air-dry till all the moisture content was removed and gold coated by Polaron, SEM Coating System to 3A° for 1.3 min and observed in LEO 435VP Scanning Electron Microscope at 2000× and 30, 000× magnification.

6.2.4 SDS-PAGE Analysis of Occlusion Bodies (OBs) of BmNPV

The purified OB suspension with 5×10^7 OBs/mL was mixed with sample buffer (125 mM Tris-HCl, pH 6.7; 4% SDS; 30% glycerol; 0.002% bromophenol blue and 10% β-mercaptoethanol) and boiled for 5 min at 95 °C and separated on 12% resolving gel at 150 V for 1 h.

6.2.5 SDS-PAGE Profile of BmNPV-Infected Hemolymph Proteins

The SDS-PAGE of BmNPV-infected silkworm hemolymph and uninfected hemolymph of fifth instar day 1 to day 9 was collected aseptically. 10 μL of hemolymph samples was denatured in sample buffer (1.5 M Tris-HCl, pH 6.8; 25% glycerol; 15% β-mercaptoethanol; 5% SDS; 0.002% bromophenol blue) and boiled at 95 °C for 5 min. Hemolymph proteins were resolved in 12% polyacrylamide gel and silver stained.

6.2.6 Two-Dimensional Electrophoresis of BmNPV-Infected Silkworm Hemolymph Proteins

Fifth instar day 1 to day 7 of BmNPV-infected silkworm hemolymph proteins was analyzed by using two-dimensional gel electrophoresis. The hemolymph of BmNPV-infected and control hemolymph solubilize in lysis buffer (7 M urea; 2 M thiourea; 4% CHAPS; 0.2% ampholyte. pH 5–8 (Sigma-Aldrich); 1 mM PMSF; 2 mM EDTA; 65 mM DTT. pH 8.5).

The isoelectric focusing (IEF) electrophoresis was performed according to O'Farrell (1975). The 2-DE was performed in glass capillary tubes; tubes were loaded with the first dimension capillary tube gel monomer solution (9.2 M urea; 4.5% acrylamide/bis-acrylamide; 5% ampholyte, pH 5–8; 5% IGEPAL CA-630 electrophoresis detergent; make it to 20 mL with Milli-Q water, 40 μL of 10% ammonium persulfate, and 20 μL TEMED) after polymerization; it was overlaid with gel overlaying solution (GOS) (9 M urea; 1% ampholyte, pH 5–8 (Sigma-Aldrich)), and the remaining space was filled with upper tank buffer (20 mM NaOH). The lower tank was filled with lower tank buffer (7.8 M orthophosphoric acid).

The IEF gel was pre-run with a constant voltage of 200 V for 15 min, 300 V for 30 min, and 500 V for 30 min. After pre-run, pre-estimated solubilized hemolymph protein samples of 100 μg were loaded in each tube. On the protein samples, GOS and upper tank buffer were filled. Electrophoresis was performed with constant voltage of 600 V for 12 h and 700 V for 1 h at room temperature.

The gels after the first dimension electrophoresis were retrieved from the glass tubes. Then the second dimension SDS-PAGE was performed in 12% polyacrylamide gel at 150 V. Protein spots on the gels after electrophoresis were visualized by silver staining.

6.3 BmNPV Infection-Driven Proteomic Changes in Hemolymph of Silkworm *Bombyx mori* L.

Nuclear polyhedrosis virus infection in silkworm *Bombyx mori* results in several physical, biological, physiological, and molecular changes in the host. The main aim behind these changes is to ensure successful viral infection and replication (Fig. 6.1).

Silkworms with BmNPV infection showed intersegmental swelling (Fig. 6.2); they stop feeding due to loss of appetite which is observed as empty gut anatomically. As the disease progresses, larval hemolymph turned mild yellowish to milky white with reduced viscosity in comparison to healthy silkworm hemolymph which is more viscous and transparent (Figs. 6.3 and 6.4).

The isolation of the virus, multiplication, and microscopic observations of OBs in the infected hemolymph confirmed the virus to be *Bombyx mori* nuclear polyhedrosis virus. Similar disease symptoms and characteristics of the virus were reported in grasserie-infected silkworms (Palhan and Gopinathan 1996).

The partial purification of virus by centrifugation and washing with distilled water and microscopic observations revealed billions of colorless, crystalline, highly refractive, polyhedron-shaped OBs. The size of the OBs was approximately 0.5–2 μm (Fig. 6.5). The sucrose density gradient purification of OBs of BmNPV is segregated as mature OBs settled at the bottom, and immature were dispersed at 55–65% of sucrose gradient in the centrifuge tube (Fig. 6.8). This methodology is effective to yield pure OBs for electron microscopy and proteomic studies (Liu et al. 2008).

Scanning electron microscopy of BmNPV isolate revealed OBs with regular polyhedron shape with significant variations in shape. The SEM at the magnification of 30,000× showed that the OBs were 1.5–3 μm in size and in addition to polyhedron-shaped OBs; small circular OBs (yellow arrows) and cubic OBs (green arrows) were also observed (Fig. 6.7).

The ODVs isolated from milky white suspension of OBs were incubated with polyhedra lysis buffer (pH 10.5) at 37 °C for 2 h; suspension became colorless due to the breakdown of proteinaceous polyhedrin protein and release of ODVs. The liberated ODVs were further purified by using sucrose density gradient centrifugation and pure ODVs were collected at 50–55% gradient of sucrose was collected (Fig. 6.6).



Fig. 6.1 Grasserie-infected and healthy silkworms



Fig. 6.2 BmNPV-infected silkworm showing fragile skin

The major protein of the NPV occlusion body is polyhedrin, which is highly conserved of all baculovirus proteins. Initiation and formation of polyhedron-shaped occlusion bodies are believed to require specific protein-protein interactions between polyhedrin and proteins in the virion envelope (Russell and Rohrmann 1990). The OBs of BmNPV are commonly polyhedral in shape, but mutations in polyhedrin gene and some other genes may affect OBs morphogenesis. The shape of OBs may be tetrahedral, hexahedral, or octahedral in BmNPV are reported (Bilimoria 1991). The genetic difference is responsible for variations in the formation of OBs of BmNPV. A recent study confirmed the mutation in the genome of BmNPV leads to the formation of cubic OBs, in addition to the single point mutation in polyhedrin gene (Lin et al. 2000); baculovirus repeated ORFs (bro) and homologous repeat (HR) regions showed the major differences in genome size of the normal and cubic strains of BmNPV (Cheng et al. 2012) (Fig. 6.7).

For the proteomic studies of OBs, sucrose density gradient purified OBs and SDS-PAGE analysis revealed 13 predominant bands on 12% polyacrylamide gel (Fig. 6.8). The molecular weight of all the bands was predicted by comparing with medium range protein marker with 98–29 kDa range, and our results are also in confirmation with previous studies (Perera et al. 2007; Braunagel and Summers

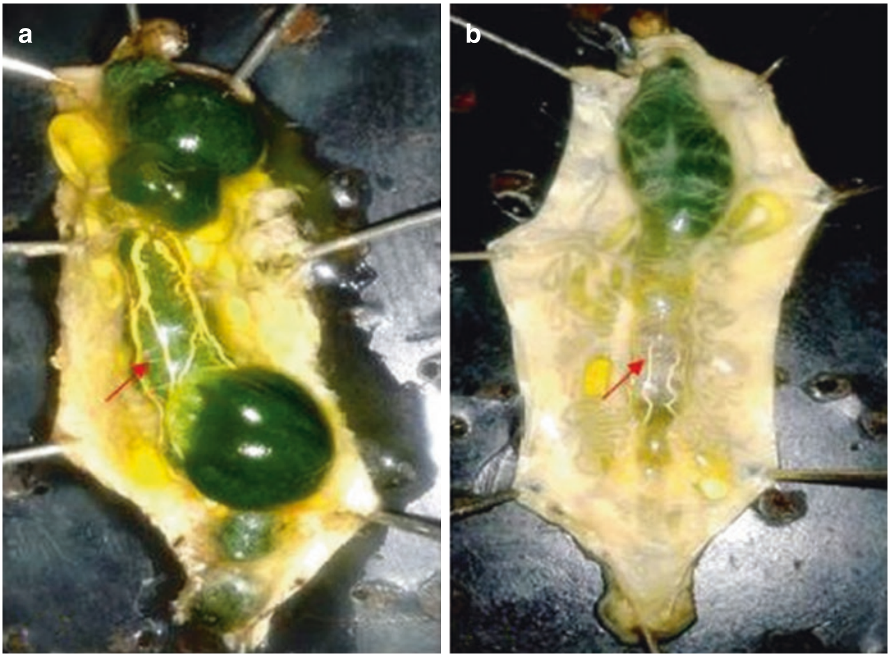


Fig. 6.3 Anatomy of (**a**) healthy silkworm showing mulberry leaves the in gut. (**b**) BmNPV-infected silkworm showing empty gut

Fig. 6.4 (**a**) BmNPV-infected hemolymph. (**b**) Control hemolymph



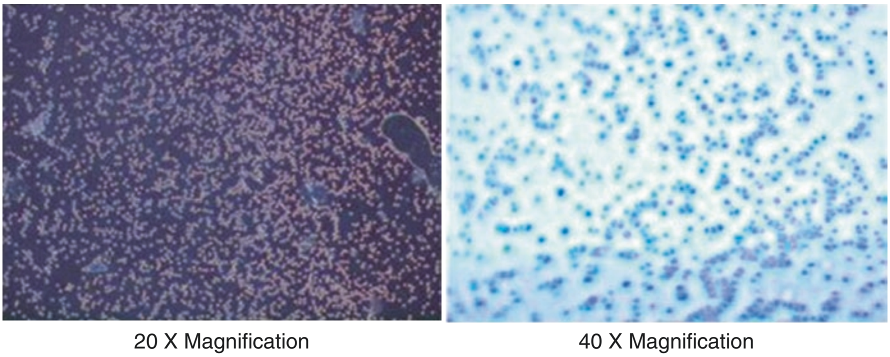


Fig. 6.5 Microscopic image of occlusion bodies (OBs) in 20× and 40× magnifications

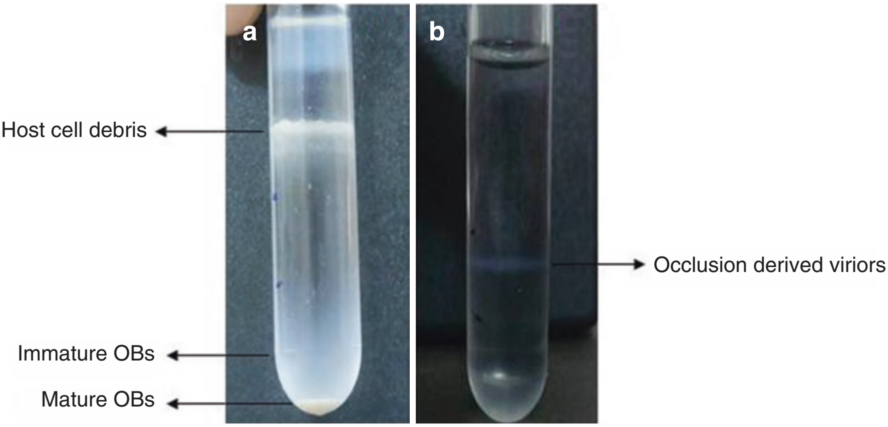


Fig. 6.6 Sucrose density gradient purification of (**a**) BmNPV OBs and (**b**) ODVs

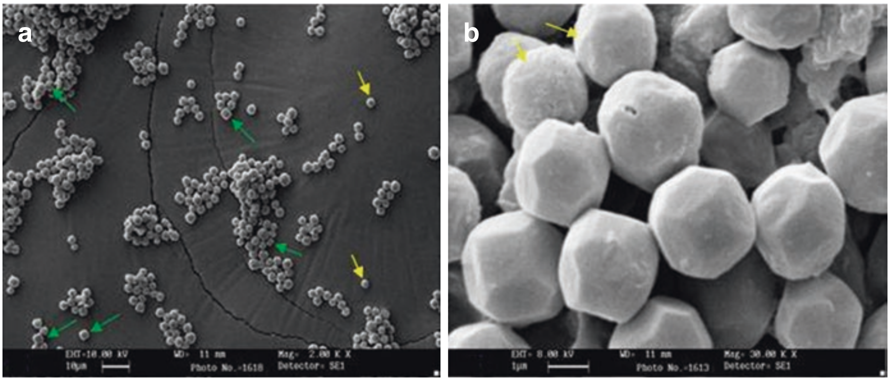


Fig. 6.7 Scanning electron micrographs of OBs (**a**) 2000× magnification and (**b**) 3000× magnification

2007). Interestingly, the protein of ODV-E56 with molecular weight of 41.3 kDa, BV/ODV-C42 with molecular weight of 41.6 kDa, GP41 with molecular weight of 44.9 kDa, and ODV-E66 with molecular weight of 79.2 kDa protein bands appeared with an unusual molecular weight than predicted range on the gel due to the fragmentations of the respective proteins during analysis (Table 6.1). In previous studies, those bands were analyzed and confirmed by MALDI-TOF and MS/MS studies and amino acid sequencing (Deng et al. 2007; Omaththage et al. 2007; Braunagel and Summers 2007).

Viral proteins (VPs) play a very important role in determining the virus ability to interact with the host tissues to enable the entry of viral DNA into host genome. Study of VPs is crucial in this direction.

SDS-PAGE protein profile of BmNPV-infected fifth instar silkworm hemolymph profile showed significant differences as overexpression (white ellipse), underexpression (yellow ellipse), no expression (red ellipse), and new expression (white

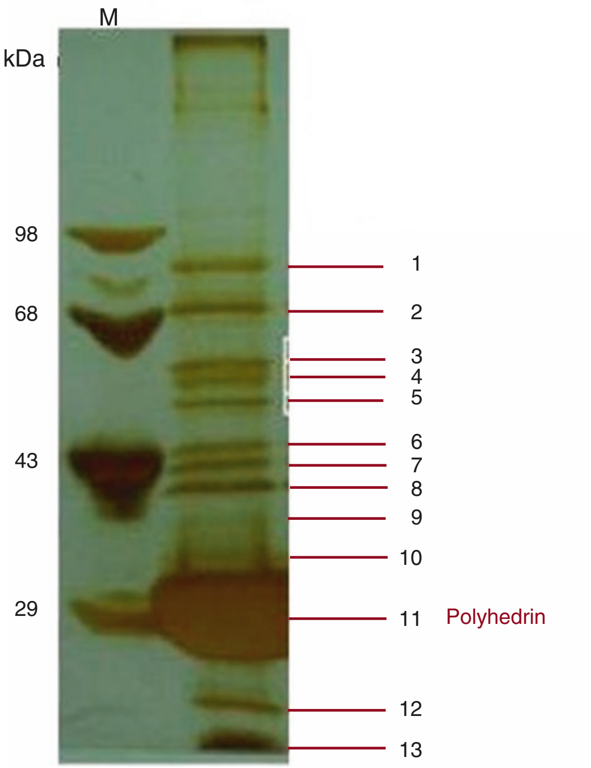


Fig. 6.8 SDS-PAGE profiling of OBs, *M* medium range protein marker

arrow) of many protein bands with reference to control hemolymph proteins of respective days in fifth instar larvae.

The progressive underexpression of 30 kDa group of lipoproteins in 5th, 6th, and 7th days of postinfection was observed (Fig. 6.9, Lanes 10, 12, and 14). This group of proteins is the major hemolymph proteins which include the storage proteins (Kishimoto et al. 1999). They are involved in inhibiting apoptosis, also in self-defense of host insect (Ujita et al. 2005). Generally BmNPV-infected silkworms show loss of body weight due to secondary infection with bacterial flacherie; hence, body turns black prior to death. This might be due to failed immune response and apoptosis of infected cells. This may be due to the underexpression of major hemolymph proteins of 30 kDa. Our results are in confirmation with the diagnostics of disease as noticed in the infected larvae as well as the protein profile analysis. The storage hemolymph proteins of ~75–80 kDa are moderately expressed in fifth, sixth, and seventh dpi in infected hemolymph as compared to healthy hemolymph (Fig. 6.9, Lanes 10, 12, and 14).

Silkworms possess two kinds of storage proteins (SP), methionine-rich SP1 and arylphorin-rich SP2. These proteins are prominent in all the five instars of silkworm hemolymph. Prior to pupation, storage proteins are taken up by the fat bodies and used to form somatic tissues and female reproductive products of pupa (Nagata and Kobayashi 1990). The reduction in the expression of storage proteins might be the responsible factor for delay in pupation of BmNPV-infected silkworms.

Table 6.1 Structural proteins of OBs and ODVs on 12% polyacrylamide gel of SDS-PAGE

Band no.	Predicted size from SDS-PAGE (kDa)	Protein name	Number of amino acids (aa)	BmNPV ORF	Function
1	79.9	VP80	692	BmNPV orf88	Capsid protein
2	74	P74	645	BmNPV orf115	Oral infectivity
3	44.9	GP41	403	BmNPV orf66	Tegument protein
4	79.2	ODV-E66	702	BmNPV orf37	ODV envelope
5	59.8	PIF-1	527	BmNPV orf97	*per os* infectivity of NPVs, ODV envelope protein
6	55.5	P49	476	BmNPV orf118	ODV associated protein
7	41.6	BV/ODV-C42	362	BmNPV orf85	Regulating the cell cycle and enhancing viral infectivity
8	45	ODV-EC43	391	BmNPV orf92	ODV protein of envelope and nucleocapsids, having role during infection
9	41.3	ODV-E56	375	BmNPV orf124	ODV envelope protein
10	44.9	GP41	403	BmNPV orf66	Tegument protein
11	28.8	Polyhedrin	245	BmNPV orf1	OB structural protein
12	25.6	ODV-E25	228	BmNPV orf77	ODV envelope
13	8.1	P6.9	65	BmNPV orf84	DNA binding protein

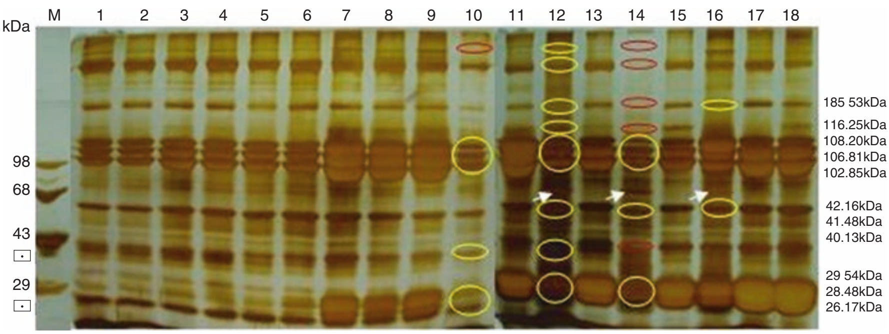


Fig. 6.9 SDS-PAGE of BmNPV-infected silkworm hemolymph proteins. *M* medium range protein marker. Lanes 1, 3, 5, 7, 9, 11, 13, 15, 17: control hemolymph of fifth instar first to ninth days postinfection. Lanes 2, 4, 6, 8, 10, 12, 14, 16, 18: BmNPV-infected hemolymph of fifth instar day 1 to day 9

The predominant band of hemolymph proteins of ~42–45 kDa is diffused in sixth, seventh, and eighth days postinfection (dpi) as compared with other infected as well as control hemolymph protein bands (Fig. 6.7, Lanes 12, 14, and 16). Further characterization of this band is required to study its role in the process of BmNPV infectivity. Interesting observation of this study was, at the region of ~70–90 kDa, a new protein band expressed in sixth, seventh, and eighth dpi infected hemolymph.

But the intensity of expression was more in sixth dpi and suddenly reduced in seventh and eighth dpi (Fig. 6.7. White arrows). On the grounds of these observations, we predicted that it may be an antiviral protein band but failed to overexpress to take over BmNPV infection due to the lack of host insect physiological state to support its expression. But further analysis is required to confirm this hypothesis.

We predicted 36 kDa protein of the aldo-keto reductase from day 1 to day 9 of control hemolymph, whereas it is progressively diffused in fifth to sixth dpi and sharply disappeared in seventh dpi of BmNPV-infected hemolymph (Fig. 6.7, Lanes 10, 12, and 14). This enzyme is actively involved in the aldo-keta reductase activity in carbohydrate metabolism of silkworm. The underexpression or suppression of aldo-keto reductase may be responsible for the interruption in normal bioenergetics of carbohydrates, failure of appetite, and other physiological activities in BmNPV-infected silkworms.

The temporal fluctuations in expression observed in ~260–270 kDa host isoforms of hemolymph proteins. In fifth dpi, these bands abruptly disappeared and mildly expressed in sixth dpi and again disappeared in seventh dpi but clearly expressed in 8th and 9th dpi. The highly expressed hemolymph protein band of ~210–230 kDa is underexpressed in sixth dpi but failed to express in seventh dpi (Fig. 6.7, Lanes 12 and 14) and normally expressed in 8th and 9th dpi. The protein expression pattern in case of ~185 kDa protein band is underexpression at sixth dpi but not expressed in 7th dpi and again poorly expressed in 8th dpi and normally expressed in 9th dpi of BmNPV-infected hemolymph (Fig. 6.7, Lanes 16 and 18).

The silkworm has an open circulatory system, and hemolymph is the main circulating fluid in the hemocoel of the silkworm. It bathes all tissues and organs in the insect body and transports nutrients, hormones, and metabolic wastes (Gilbert and Chino 1974). The hemolymph proteins represent the products of gene expression. Their levels show generic-specific, tissue-specific, and instar-specific variations during insect metamorphosis (Hou et al. 2010). The hemolymph of silkworm acts as a reservoir for many proteins that includes juvenile hormone-binding proteins, RNA-binding proteins, paralytic peptide-binding protein, aldose reductase, low molecular weight lipoproteins, carboxylesterases, zinc finger proteins, imaginal disk growth factor, gelsolin, glyoxylate reductase, hydroxypyruvate isomerase, aminoacylase, trypsin inhibitor, transferrin protein, serine proteases, chymotrypsin inhibitor, hemolin, prophenoloxidase, 30 kDa lipoproteins, instar-specific proteins, *N*-acetylglucosaminidase, and many other unidentified proteins (Hou et al. 2010).

These proteins and enzymes are associated with silk formation, hemocyte production, ecdysis, eclosion, metabolism, metamorphosis, immunity, locomotion, cocoon spinning, digestion, respiration, tissue degeneration, organ growth, and from heat shock control to gene expression (Li et al. 2006; Choi et al. 2008; Nakahara et al. 2009). The hemolymph proteins represent 93 silk gland proteins, 177 fat body proteins, and 278 skeletal muscle proteins (Takasu et al. 2005; Kyung et al. 2006; Zhang et al. 2007).

The electrophoretic analysis of hemolymph proteins of NPV-infected silkworm shows the reduction in all the protein fractions (Watanabe 1986). The level of amino acid transferases and parasitic polypeptides increases in NPV-infected insects and decreases in host cellular polypeptides (Kobayashi et al. 1990). BmNPV infection

decreases the activity of digestive enzymes in the midgut of silkworm larvae (Gururaj et al. 1999).

Further proteomic studies on hemolymph proteins by two-dimensional gel electrophoresis protein profiling of fifth instar day 1 to day 7 silkworm hemolymph proteins revealed the expression of number of discrete protein spots within the pI of 5–8 in BmNPV-infected and control hemolymph (Table 6.2). The 2-DE of hemolymph profile showed considerable variations in expression patterns as overexpression (white ellipse), underexpression (yellow ellipse), no expression (red ellipse), and new expression (white arrow) of some of the protein spots with reference to control hemolymph proteins of respective dpi.

The comparative proteomics of fifth instar day 1 control and BmNPV-infected hemolymph proteins observed the expression of nine highly intense new protein spots and inhibited expression of 25–30 kDa proteins, which are abundantly expressed in control hemolymph. The comparison of protein profiling was confirmed with previously reported studies, and 12 protein spots were matched (Li et al. 2006). One of the spots is predicted as ribosomal protein, which is actively involved in elongation process during translation. In BmNPV-infected silkworms, all the physiological functions are affected, because of the inhibition in the expression of ribosomal proteins, directly affecting the mechanism of protein synthesis.

In the second dpi 2-DE pattern of expression, there are no visible changes in the proteome maps of control and infected hemolymph proteins. We predicted two protein spots by comparing with the earlier studies (Li et al. 2006). The predicted proteins are HAD-type hydrolase/phosphatase and haloacid dehalogenase (HAD)-like hydrolase, which consists mainly of uncharacterized enzymes with phosphatase, beta-phosphoglucomutase, and dehalogenase-like activities, and are also involved in the hydrolysis of nucleotides, phosphoglycolate, phosphoserine, pyridoxal, etc.

In third dpi studies, in control hemolymph, nearly 60 protein spots were clearly visible; out of them, nearly 38 protein spots were identical to previously studied proteome map of hemolymph proteins (Li et al. 2006; Kajiwara et al. 2009; Hou et al. 2010). The group of 30 kDa proteins is strongly expressed. But in BmNPV-infected hemolymph 2-DE profile, we were unable to resolve the expected proteins around isoelectric point 5 (pI 5). The protein spots in the region of pI 6 were overexpressed in addition to the expression of new protein spots (Fig. 6.10).

Table 6.2 2-DE number of protein spots observed in control and infected hemolymph proteins in 1st to 8th dpi of BmNPV infection

Days of post infection in 5th instar larvae	Protein spots in control hemolymph	Protein spots in infected hemolymph
1st day	66	63
2nd day	49	38
3rd day	60	50
4th day	37	30
5th day	48	21
6th day	30	11
7th day	29	17
8th day	33	29

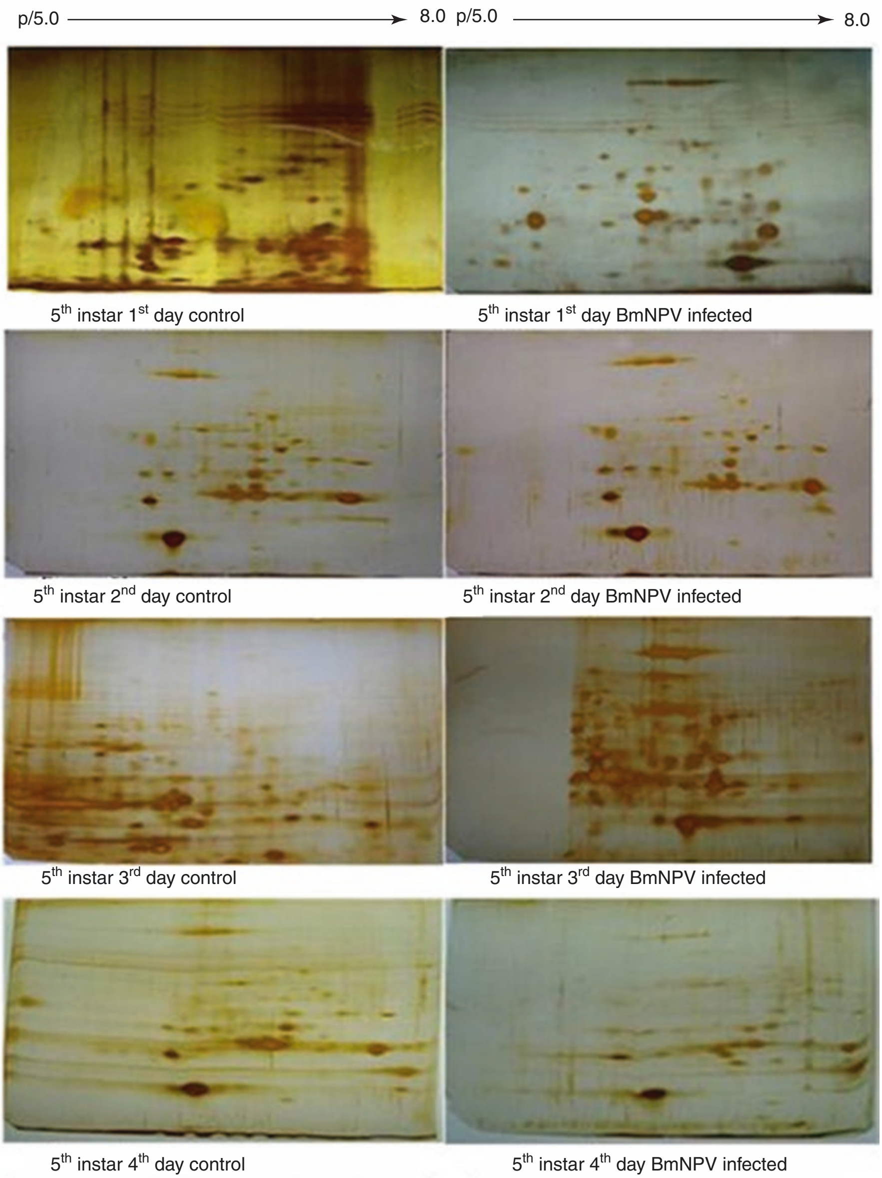


Fig. 6.10 2-DE profile of BmNPV-infected silkworm hemolymph proteins of fifth instar first day to fourth days postinfection

In fifth instar day 4 proteome of control and BmNPV-infected hemolymph proteins dictates, sudden underexpression of many proteins in 30 kDa range was noticed. Some of the protein spots present at pI 6 are abruptly underexpressed in BmNPV-infected hemolymph. The spot of HAD-type hydrolase/phosphatase enzyme is normally expressed as compare to the control (Fig. 6.10).

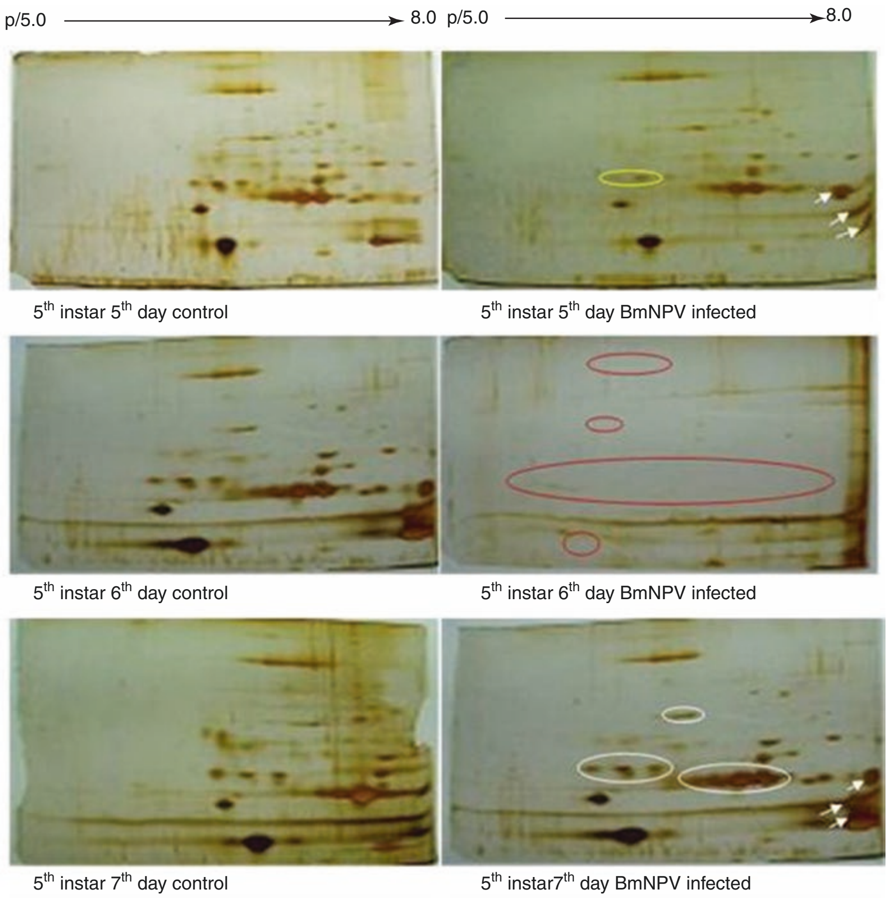


Fig. 6.11 2-DE profile of BmNPV-infected silkworm hemolymph proteins at fifth instar fifth day to seventh days postinfection

The day 5 2-DE profiling showed underexpression of 30 kDa lipoproteins in BmNPV-infected hemolymph. Some protein spots are constantly expressed as usual with slight decoration variations. The intensely expressed spots with pI 6 were underexpressed. As in day 4 of infected hemolymph, where we noticed three newly expressed predicted spots of polyhedrin at pI 7.8 (Fig. 6.11).

The day 6 2-DE profiling of BmNPV infection in comparison with the control revealed drastic underexpression of all the protein spots excluding predicted polyhedrin spot in BmNPV-infected hemolymph proteins. This kind of unusual pattern of profiling may be due to hemolymph collected from intensely infected silkworm with less quantity of hemolymph and more OBs (Fig. 6.11).

The day 7 protein profiling revealed overexpression of three protein spots and underexpression of two spots at 30–45 kDa and pI 5.8–6.8 in BmNPV-infected hemolymph as compared to control hemolymph. A specific protein spot expressed

intensely with different decoration and three new protein spots were overexpressed (arrow mark). The enzyme alcohol dehydrogenase II is related to the fat biosynthesis at fifth instar stage in preparation for pupation, and transcriptional factor is essential for transcription. These two proteins are underexpressed in BmNPV-infected hemolymph which adversely affects fat biosynthesis and transcription in BmNPV-infected silkworms.

Conclusion

Silkworms have emerged as new tools for biotechnological studies as model animal and bioreactor. In addition to genomics, proteomics, and metabolomics studies on silkworms, basic studies like infectious diseases of silkworms, mode of infections, disease establishment, host-pathogen interactions, and resistance of the host toward the infection of entomopathogens are scarce. All these studies are incomplete without authentic knowledge of relevant proteins which are the ultimate biomolecules to decide most of the biological functions. Though a lot of information on crystal structure, catalytic activity, and the functions of proteins have been elucidated, the role of several proteins still remains unresolved.

The biological functioning of all the organisms not only depends on static genome but the dynamic population of proteins determined by an interplay of gene and protein regulation with extracellular influences. By using genome sequence data, active domains of functional proteins, posttranslational modifications, cannot be predicted. To know the functional feature of proteins, posttranslational modifications like phosphorylation, glycosylation, ubiquitination, and methylation are very important. There is increasing interest in the field of proteomics, the large-scale study of proteins as a complement to genomics, and functional genomics is emerging.

The silkworm host and baculoviral interaction studies by using SDS-PAGE in fifth instar day 1 to day 9 in BmNPV-infected silkworms with reference to healthy silkworm hemolymph revealed the downregulation of housekeeping, host-specific hemolymph proteins, and upregulation of BmNPV-specific structural proteins, specifically polyhedrin. In contrast to this, a new protein band of ~70–90 kDa is identified in sixth, seventh, and eighth dpi of BmNPV predicted to be an antiviral protein. Two-dimensional gel electrophoresis of BmNPV-infected fifth instar day 1 to day 7 in contrast to healthy hemolymph proteins dictates downregulation of host-specific proteins which are responsible for all the physiological activities of the host and overexpression of pathogen-derived and pathogen-induced proteins in the late phase of infection. We observed nearly 70 discrete protein spots and predicted their names and role in host-pathogen interactions by comparing with the available protein data base of hemolymph proteins.

These research findings are clearly evident in confirming the changes of host protein profiles which mostly favor the disease course and give the pathogen a very proven strategy. Our understanding from the present study will help us to elucidate much more detailed approach to host-pathogen interactions in silkworms.

References

Belyavskyi M, Braunagel SC, Summers MD (1998) The structural protein ODV-EC27 of *Autographa californica* nucleopolyhedrovirus is a multifunctional viral cyclin. Proc Natl Acad Sci U S A 95(19):11205–11210

Bilimoria SL (1991) The biology of nuclear polyhedrosis viruses. In: Kurstak E (ed) Viruses of invertebrates. Marcel Dekker, New York, pp 1–72. 360p

Braunagel SC, Elton DM, Ma H, Summers MD (1996) Identification and analysis of an *Autographa californica* nuclear polyhedrosis virus structural protein of the occlusion-derived virus envelope: ODV-E56. Virology 217:97–110

Braunagel SC, Summers MD (2007) Molecular biology of the Baculovirus occlusion-derived virus envelope. Curr Drug Targets 8:1084–1095

Cheng R-L, Xu Y-P, Zhang C-X (2012) Genome sequence of a *Bombyx mori* nucleopolyhedrovirus strain with cubic occlusion bodies. J Virol 86(18):10245

Choi SS, Rhee WJ, Park TH (2008) Inhibition of human cell apoptosis by silkworm hemolymph. Biotechnol Prog 18:874–878

Clemens MJ, Elia A (1997) The double stranded RNA-dependent protein kinase PKR: structure and function. J Interf Cytokine Res 17(9):503–524

Deng F, Wang R, Fang M, Jiang Y, Xu X, Wang H, Chen X, Arif BM, Lin G, Wang H, Zhihong H (2007) Proteomics analysis of *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus identified two new occlusion-derived virus-associated proteins, HA44 and HA100. J Virol 81(17):9377–9385

Du X, Thiem SM (1997) Characterization of host range factor 1(hrf-1) expression in *Lymantria dispar* Multinucleopolyhedrovirus and recombinant *Autographa californica* infected IPLB-Ld652Y cells. Virology 227:420–430

Engelhard EK (1994) The insect tracheal system: a conduit for the systemic spread of *Autographa californica* multinuclear polyhedrosis virus. Proc Natl Acad Sci U S A 91:3224–3227

Fang M, Dai X, Theilmann DA (2007) *Autographa californica* multiple nucleopolyhedrovirus EXON0 (ORF141) is required for efficient egress of nucleocapsids from the nucleus. J Virol 81(18):9859–9869

Gilbert LI, Chino H (1974) Transport of lipids in insects. J Lipid Res 15:439–456

Goley ED (2006) Dynamic nuclear actin assembly by Arp2/3 complex and a baculovirus WASP-like protein. Science 314(5798):464–467

Granados RR, Lawler KA (1981) In vivo pathway of *Autographa californica* baculovirus invasion and infection. Virology 108:297–308

Gross CH, Russell RLQ, Rohrmann GF (1994) The *Orgyia pseudotsugata* baculovirus p10 and polyhedron envelope protein genes: analysis of their relative expression levels and role in polyhedron structure. J Gen Virol 75:1115–1123

Gururaj CS, Sekharappa BM, Sarangi SK (1999) Effect of BmNPV infection on the digestive enzyme activity in the silkworm, *Bombyx mori* L. Indian J Seric 38(2):102–106

Hou Y, Zou Y, Wang F, Gong J, Zhong X, Xia Q, Zhao P (2010) Comparative analysis of proteome maps of silkworm hemolymph during different developmental stages. Proteome Sci 8(45):1–10

Kajiwara H, Imamaki A, Nakamura M, Mita K, Xia Q, Ishizaka M (2009) Proteome analysis of silkworm 2. Hemolymph. J Electrophor 53:27–31

Kishimoto A, Nakato H, Izumi S, Tomino S (1999) Biosynthesis of major plasma proteins in the primary culture of fat body cells from the silkworm, *Bombyx mori*. Cell Tissue Res 297: 329–335

Kobayashi M, Kotake M, Sugimori H, Nagamine T, Kajiura Z (1990) Identification of virus specific polypeptides and translatable mRNAs in the isolated pupal abdomens of the silkworm, *Bombyx mori* infected with nuclear polyhedrosis. J Invertebr Pathol 55(1):52–60

Kyung HS, Su JJ, Young RS, Seok WK, Sung SH (2006) Identification of up-regulated proteins in the haemolymph of immunized *Bombyx mori* larvae. Comp Biochem Physiol D1:260–266

Lehane MJ, Aksoy S, Levashina (2004) Blood immune responses and parasite transmission in insects. Trends Parasitol 29(9):433–439

Lepore LS, Roelvink PR, Granados RR (1996) Enhancin, the granulosis virus protein that facilitates nucleopolyhedrovirus (NPV) infections, is a metalloprotease. J Invertebr Pathol 68(2):131–140

Li X-h, Wu X-f, Yue W-f, Liu J-m, Li G-l, Miao Y-g (2006) Proteomic analysis of the silkworm (*Bombyx mori* L.) Hemolymph during developmental stage. J Proteome Res 5(10):2809–2814

Lim DS, Ko SH, Kim SJ, Park YJ, Park JH, Lee WY (2002) Photoinactivation of vesicular stomatitis virus by a photodynamic agent, chlorophyll derivatives from silkworm excreta. J Photochem Photobiol B 67(3):149–156

Lin GY, Zhong J, Wang XZ (2000) Abnormal formation of polyhedra resulting from a single mutation in the polyhedrin gene of *Autographa californica* multicapsid nucleopolyhedrovirus. J Invertebr Pathol 76(1):13–19

Liu X, Chen K, Cai K, Yao Q (2008) Determination of protein composition and host-derived proteins of *Bombyx mori* Nucleopolyhedrovirus by 2-dimensional electrophoresis and mass spectrometry. Intervirology 51:369–376

McCarthy CB, Theilmann DA (2008) AcMNPV ac143 (odv-e18) is essential for mediating budded virus production and is the 30th baculovirus core gene. Virology 375:277–291

Murphy FA, Fauquet CM, Bishop DHL, Ghabrial SA, Jarvis AW, Martelli GP, Mayo MA, Summers MD (1995) Virus taxonomy. Classification and nomenclature of viruses. Sixth Report of the International Committee on Taxonomy of Viruses. Springer, Wien New York, Arch Virol, 10

Nagata M, Kobayashi M (1990) Quantitative changes in storage proteins during larval development of the silkworm, *Bombyx mori*. J Seric Sci Jpn 59:461–468

Nakahara Y, Shimura S, Ueno C, Kanamori Y, Mita K, Kiuchi T, Kamimura M (2009) Purification and characterization of silkworm hemocytes by flow cytometry. Dev Comp Immunol 33:439–448

Nakazawa H, Tsuneishi E, Ponnuvel KM, Furukawa S, Asaoka A, Tanaka H, Ishibashi J, Yamakawa M (2004) Antiviral activity of a serine protease from the digestive juice of *B. mori* larvae against NPV. Virology 321(1):154–162

Nie Y, Fang M, Theilmann DA (2009) AcMNPV AC16 (DA26, BV/ODV-E26) regulates the levels of IE0 and IE1 and binds to both proteins via a domain located within the acidic transcriptional activation domain. Virology 385(2):484–495

Ohkawa T, Volkman LE, Welch MD (2010) Actin-based motility drives baculovirus transit to the nucleus and cell surface. J Cell Biol 190(2):187–195

O'Farrell PH (1975) High resolution two-dimensional electrophoresis of proteins. Journal of biological chemistry 250(10):4007–4021

Palhan VB, Gopinathan KP (1996) Characterization of a local isolate of *Bombyx mori* nuclear polyhedrosis virus. Curr Sci 70(2):147–153

Perera O, Green T B, Stevens S M, White S, Becnel JJ (2007) Proteins associated with Culex nigripalpus nucleopolyhedrovirus occluded virions. Journal of virology 81(9):4585–4590

Ponnuvel KM, Nakazawa H, Furukawa S, Asaoka A, Ishibashi J, Tanaka H, Yamakawa M (2003) A lipase isolated from the silkworm shows antiviral activity against NPV. J Virol 77(19):10725–10729

Prudhomme JC, Couble P (2002) Perspectives in silkworm transgenesis. Curr Sci 83(4):432–438

Rohrmann GF (2011) Baculovirus Molecular Biology, 2nd edn. National Center for Biotechnology Information (US), Bethesda

Russell RLQ, Rohrmann GF (1993) A 25 kilo dalton protein is associated with the envelopes of occluded baculovirus virions. Virology 195:532–540

Russell RLQ, Funk CJ, Rohrmann GF (1997) Association of a baculovirus encoded protein with the capsid basal region. Virology 227:142–152

Russell RLQ, Rohrmann GF (1990) A baculovirus polyhedron envelope protein: immunogold localization in infected cells and mature polyhedra. Virology 174:177–184

Takasu Y, Yamada H, Saito H, Tsubouchi K (2005) Characterization of *Bombyx mori* sericins by the partial amino acid sequences. J Insect Biotechnol Sericol 74:103–109

Terenius O (2004) Anti-parasitic and anti-viral immune responses in insects. Ph. D Thesis. Department of Genetics, Microbiology and Toxicology, Stockholm University, Stockholm

Ujita M, Katsuno Y, Kawachi I, Ueno Y, Banno Y, Fujii H, Hara A (2005) Glucan-binding activity of silkworm 30-kDa apolipoprotein and its involvement in defense against fungal infection. Biosci Biotech Bioch 69:1178–1185

Vanarsdall AL, Pearson MN, Rohrmann GF (2007) Characterization of baculovirus constructs lacking either the Ac 101, Ac 142, or the Ac 144 open reading frame. Virology 367:187–195

Vigdorovich V, Miller AD, Strong RK (2007) Ability of hyaluronidase 2 to degrade extracellular hyaluronan is not required for its function as a receptor for jaagsiekte sheep retrovirus. J Virol 8(7):3124–3129

Wang P, Granados RR (1997) An intestinal mucin is the target substrate for a baculovirus enhancin. Proc Natl Acad Sci U S A 94(13):6977–6982

Wang R (2010) Proteomics of the *Autographa californica* Nucleopolyhedrovirus budded Virions. J Virol 84:7233–7242

Wang Y (1995) Interaction of p53 with its consensus DNA-binding site. Mol Cell Biol 15(4):2157–2165

Watanabe H (1986) Resistance of the silkworm, *Bombyx mori* to viral infections. Agric Ecosyst Environ 15(2–3):131–139

Wu W (2006) *Autographa californica* multiple nucleopolyhedrovirus nucleocapsid assembly is interrupted upon deletion of the 38K gene. J Virol 80(23):11475–11485

Xu H (2008) *Bombyx mori* nucleopolyhedrovirus ORF56 encodes an occlusion-derived virus protein and is not essential for budded virus production. J Gen Virol 89:1212–1219

Yang S, Miller LK (1998) Expression and mutational analysis of the baculovirus very late factor 1 (vlf-1) gene. Virology 245(1):99–109

Zhang JH (2005) Effects of Ac150 on virulence and pathogenesis of *Autographa californica* multiple nucleopolyhedrovirus in noctuid hosts. J Gen Virol 86(6):1619–1627

Zhang P, Aso Y, Jikuya H, Kusakabe T, Lee JM, Kawaguchi Y, Yamamoto K, Banno Y, Fujii H (2007) Proteomic profiling of silkworm skeletal proteins during larval-pupal metamorphosis. J Proteome Res 6:2295–2303

Part II

Molecular Based Studies of Insect Pathology

7 Analysis of the Viral Lytic Polysaccharide Monooxygenase Fusolin and Its Potential Application to Pest Control

Wataru Mitsuhashi

Abstract

Although microbial insecticides are generally safe for vertebrates, plants and the environment, their use has been very limited, mainly because their cost of pest control is much higher than that of chemical insecticides. To expand the use of microbial insecticides, their ability to kill pests needs to be strengthened. Increased activity will reduce the amount applied per unit area and the cost of pest control. The protein fusolin that is produced by the insect viruses entomopoxviruses and baculoviruses strongly synergistically increases the infectivity of insect viruses. Recent studies further elucidated the synergistic effect of the protein on the insecticidal activity of major entomopathogenic bacterium *Bacillus thuringiensis*. Furthermore studies have revealed that fusolin is a lytic chitin monooxygenase, and thus the mechanism of increase in the infectivity and insecticidal activity by fusolin has been elucidated in detail. These advances have expanded the possible practical applications of this protein to pest control and suggest its potential for use in a new field, namely, the development of technologies for efficient biofuel production from biomass such as chitin.

7.1 Introduction

Wide use of microbial insecticides in agriculture and forestry has been anticipated, because they are considered safe for vertebrates (including humans and livestock), plants and the environment. However, the sales of microbial insecticides constitute

W. Mitsuhashi
Institute of Agrobiological Sciences, National Agriculture and Food Research Organization, Tsukuba, Ibaraki 305-8634, Japan
e-mail: mitsuhas@affrc.go.jp

D. Kumar, C. Gong (eds.), *Trends in Insect Molecular Biology and Biotechnology*, https://doi.org/10.1007/978-3-319-61343-7_7

only a very small fraction of the total insecticide market, especially in developed countries. The main reasons for their limited use are as follows:

1. The commercial production costs of microbial insecticides are usually much higher than those of chemical insecticides, because production of the former is generally much more labour-intensive.
2. The spectrum of pests targeted by each microbial insecticide is generally narrow, so it may be necessary to use a different microbial insecticide for each insect species or group.
3. The period from infection of pests with microbes to pest death is generally longer than that with chemical pesticides, and thus they continue to consume crops.
4. Microbes are easily inactivated in the field by abiotic factors such as ultraviolet radiation and high temperature.
5. Application of microbial insecticides must be carefully timed to provide the optimal effect, mainly because the susceptibility of larvae rapidly decreases with each instar.

However, if a drastic reduction in the cost of pest control can be achieved, this may lead to expansion of the use of microbial insecticides. Therefore, attempts have been made to strengthen the ability of microbes or the toxic proteins produced by some entomopathogenic bacteria such as *Bacillus thuringiensis* (Bt) to kill pests. A major approach to achieving this goal is the use of synergistic agents that can enhance the peroral infectivity of microbes or insecticidal activity of toxins, thus reducing the amount of insecticides needed per unit area and lowering costs. More than ten synergists (synthetic compounds and proteins of insect viruses) have been found (Mitsuhashi 2009). Natural synergists are likely to be less harmful than synthetic compounds to vertebrates and the environment.

This review focuses on one of these synergists, fusolin, a lytic polysaccharide monooxygenase produced by the insect viruses entomopoxviruses (EVs) and baculoviruses. Fusolin may also potentially be used for the development of the methods of efficient conversion of biomass to chitin.

7.2 Fusolin-Producing Viruses

EVs include all viruses of the subfamily *Entomopoxvirinae* in the family *Poxviridae*. This subfamily is divided into the following three genera: *Alphaentomopoxvirus*, which infect Coleoptera; *Betaentomopoxvirus*, which infect Lepidoptera and Orthoptera; and *Gammaentomopoxvirus* of Diptera. EV virions are large, ovoid or brickshaped, and they contain a large linear double-stranded DNA (225–380 kbp) with characteristic terminal hairpin loop structures (Mitsuhashi et al. 2014b). Two types of hairpin loop sequences that are in relationship as inverted repeat are found per one EV species, and one loop links at each end of each genome molecule (Mitsuhashi et al. 2014b). EVs replicate mainly in the fat bodies of hosts.

Baculoviruses include all viruses of the family *Baculoviridae*. This family is composed of four genera: *Alphabaculovirus* which infects Lepidoptera; *Betabaculovirus*,

which infect Lepidoptera; *Gammabaculovirus*, which infects Hymenoptera; and *Deltabaculovirus*, which infect Diptera. Viruses of *Betabaculovirus* and those of the other three genera are called granuloviruses (GVs) and nucleopolyhedroviruses (NPVs), respectively. Baculoviruses infect only arthropods. Baculovirus virions are large and rod-shaped, and they contain a circular double-stranded DNA (80–180 kbp). They multiply in almost all organs and tissues of host insects.

7.3 Fusolin

Baculoviral fusolins are usually called GP37s, but in this review I refer to GP37s as fusolins. Fusolins contain 220–390 amino acid residues (aa). Their N-termini (15–20 aa) are signal peptides that are cleaved from the protein; the C-terminal regions of fusolins are poorly conserved and in general are shorter in baculoviruses than in EVs. The smallest fusolin reported so far is that of *Epinotia aporema* GV (Salvador et al. 2012).

Many baculoviruses and some EVs (and apparently metazoans) lack fusolin genes. Therefore, EVs and baculoviruses may have acquired these genes by horizontal transfer from amoebae and bacteria (Thézé et al. 2015). As EVs and baculoviruses are phylogenetically unrelated, they seem to have acquired these genes independently (Thézé et al. 2015).

7.4 Spindles

Interestingly, EV fusolins crystallise in host cells and form spindle-shaped microparticles (inclusion bodies) called spindles (long axis, 0.5–15 µm) that do not contain virus particles (Fig. 7.1). Cleavage of the signal peptide mentioned above suggests that fusolin translocates to the endoplasmic reticulum and crystallises there (Lai-Fook and Dall 2000). However, fusolins of most baculovirus species do not form spindles and are present in the cytoplasm (Phanis et al. 1999; Li et al. 2003) or nucleus (Vialard et al. 1990) of host cells. Spindles or spindle-like particles have been reported for only a few baculovirus species, e.g. *Cadra cautella* NPV, *Orgyia pseudotsugata* NPV, *Choristoneura fumiferana* defective NPV, *Ephestia kuehniella* NPV and *Galleria mellonella* GV (Adams and Wilcox 1968; Huger and Krieg 1968; Gross et al. 1993; Li et al. 2000; Chakraborty et al. 2005; Yaman et al. 2015). The following may explain the lack of spindles in baculoviruses: (1) The most common cause may be that the C-terminal regions of baculoviral fusolins contain fewer cysteines than those of EVs, and their number may not be sufficient to form intra- and intermolecular disulphide bonds, which are necessary for spindle formation (see the subsection on Mode of Action of PM Disruption). (2) An unknown host factor may be indispensable for the formation of spindles, because EV fusolins do not form spindles when produced in a baculovirus expression system in the cells of non-host insects. (3) An unknown EV factor may be important, because some NPV species do not form spindles, whereas EVs do so in the same hosts.

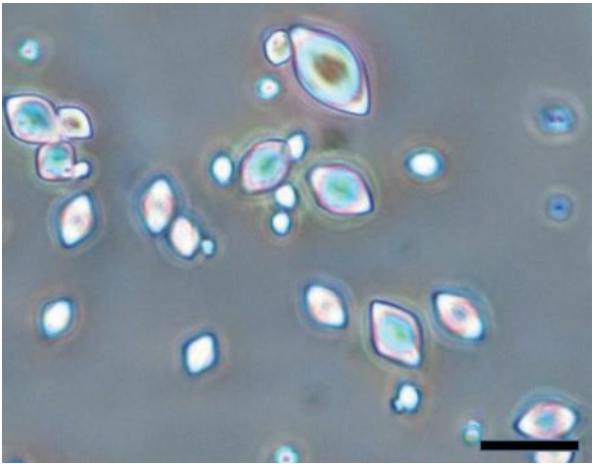


Fig. 7.1 Spindles of *Anomala cuprea* EV. They are in 12.5% sucrose solution. A photo of phase-contrast microscopy. Bar indicates 10 μm

7.5 Enhancement of Microbe Virulence

Xu and Hukuhara (1992) reported that the infectivity of polyhedra containing occluded viruses of *Pseudaletia unipuncta* NPV (PuNPV) in *Pseudaletia* (*Mythimna*) *separata* larvae (Lepidoptera) was strongly enhanced by peroral administration of spheroids—another type of EV inclusion bodies containing EV virions produced by *Pseudaletia separata* EV (PSEV)—or a solution of the spheroids. A gene responsible for this infectivity enhancement has been cloned from PSEV and has considerable sequence identity to the fusolin genes of other EV species (Hayakawa et al. 1996), indicating that this gene encodes PSEV fusolin.

Mitsuhashi et al. (1998) have found that spindles of *Anomala cuprea* EV (ACEV, genus *Alphaentomopoxvirus*) isolated from the scarabaeid beetle *A. cuprea*, but not its spheroids, strongly enhance the infectivity of *Bombyx mori* NPV (BmNPV) polyhedra in *B. mori* larvae. Wijonarko and Hukuhara (1998) have shown that not only the spheroids of PSEV but also the spindles and virions of the viruses enhance the infectivity of PuNPV polyhedra in *P. separata* larvae. However, fusolin has not been detected in ACEV spheroids (Mitsuhashi et al. 2007). Spindles of ACEV or *Helicoverpa armigera* EV enhance the infectivity of the polyhedra of other NPV species (Mitsuhashi and Sato 2000; Chakraborty et al. 2004). Fusolin of *Cydia pomonella* granulovirus produced in an *Escherichia coli* expression system enhances the infectivity of two NPV species (Liu et al. 2011). In addition, spindle-shaped bodies (diamond-shaped bodies) of *Galleria mellonella* NPV enhance the peroral infectivity of *Mythimna separata* NPV species (Chakraborty et al. 2005).

NPVs have two infectious phenotypes: the above-mentioned occluded virus (also called polyhedron-derived virus) and non-occluded virus (NOV) (budded virus). NOVs are not occluded by polyhedra and are rarely infectious to the insect midgut when administered perorally. However, Furuta et al. (2001) showed that NOVs of

BmNPV (both the wild type and polyhedrin-negative recombinant) become highly infectious perorally to *B. mori* larvae when administered with ACEV spindles. This finding suggests that spindles, together with recombinant viruses, could be a powerful tool for mass production of proteins in biofactories. Peroral inoculation of these recombinant viruses is much less labour consuming than the conventional inoculation method (injection of viruses into the host haemocoel one by one).

Baculoviral fusolin enhances the insecticidal activity of the Bt bacterium (Liu et al. 2011). EV spindles also enhance the insecticidal activity of Bt toxin alone and a commercial complete Bt formulation (Mitsuhashi et al. 2014a).

EV spindles (fusolins) may have a wide activity spectrum. For instance, ACEV spindles strongly enhance the infectivity of *Spilosoma imparilis* NPV (SiNPV) and BmNPV (Mitsuhashi et al. 1998; Mitsuhashi and Sato 2000), even though these NPVs are not very closely related taxonomically and ACEV does not infect the hosts of both NPVs. In contrast, the GV protein enhancin does not seem to have a wide spectrum of enhancement of baculovirus infection (Hukuhara et al. 1987; Derksen and Granados 1988; Goto 1990; Wang et al. 1994).

The ability of ACEV spindles to enhance NPV infectivity is highly stable against various abiotic factors, including high temperature, ultraviolet radiation and ethyl alcohol (Mitsuhashi et al. 2008). For example, ACEV spindles retain high activity even after heating at 95 °C for 30 min, whereas *Pseudaletia unipuncta* GV granules (occlusion bodies containing enhancin) are inactivated by heating at 85 °C for 10 min (Tanada 1959). Spindle stability is necessary for the use of spindles as synergists of microbial insecticides.

7.6 Natural Function of Fusolins

Enhancement of EV infectivity, as demonstrated by bioassays using ACEV spheroids combined with its spindles and recombinant ACEV fusolin produced in a baculovirus expression system, was strongly suggested to be the biological function of fusolin and spindles (Mitsuhashi et al. 2000, 2007; Mitsuhashi 2002; Takemoto et al. 2008).

Fusolin is not essential for virus replication, because recombinant *Heliothis armigera* EV with its fusolin-coding sequence replaced with that of the green fluorescent protein replicates in cell culture (Olszewski and Dall 2002). However, fusolins may be important for survival or success of these viruses in the field by enhancing their horizontal transmission between individual hosts. Spindles may be more suitable for this role than the non-crystalline form of fusolin because, as mentioned above, they are extremely stable and fusolins are abundant. Among EVs that do not produce spindles, several certainly lack the fusolin gene (Afonso et al. 1999; Bawden et al. 2000). Many baculoviruses also do not have fusolin genes (see above). It is assumed that these viruses lacking fusolin genes have an alternative means of efficiently multiplying in the field—e.g. expression of enhancin—that enhances their peroral infectivity.

7.7 Mechanism of Infectivity Enhancement

The peritrophic matrix (peritrophic membrane, PM) is an acellular membrane that lines the midgut lumen in the form of a tube, extending from the anterior midgut to the hindgut (Derksen and Granados 1988). The PM is composed primarily of chitin and proteins, including glycoproteins and proteoglycans (Wang and Granados 2001). The initial process of infection of host insects by occluded DNA viruses begins with the ingestion of occlusion bodies, which then dissolve in the alkaline midgut juices of the insects. The liberated virions then pass through the PM into the ectoperitrophic space, attach to the microvilli of the midgut columnar cells and fuse to them. Finally, the nucleocapsids of baculoviruses or the cores with lateral bodies of EVs enter the host cells (Mitsuhashi 2009). Bt protoxins are cleaved by serine proteases in the midgut to mature active forms. Then they also pass through the PM, enter the ectoperitrophic space and attach to their receptors on the microvilli of the midgut cells. The PM acts as a physical barrier against microbes or Bt toxin crystals, preventing them from reaching the microvilli (Wang and Granados 1997, 2000, 2001; Peng et al. 1999; Mitsuhashi and Miyamoto 2003; Hayakawa et al. 2004; Mitsuhashi et al. 2007, 2014a).

Mitsuhashi and Miyamoto (2003) showed that the PM of *B. mori* larvae is disintegrated by ACEV spindles and that this disintegration is accompanied by a strong increase in BmNPV infectivity; therefore, PM disintegration is thought to be the mechanism by which EV spindles enhance NPV infectivity. Likewise, the PM of *A. cuprea* larvae is disrupted after they are fed ACEV spindles, thereby facilitating infection of the host midgut by the EV (Mitsuhashi et al. 2007). This strongly suggests that mode of action of enhancement of viral infection by spindles is very similar in NPV and EV infections. Furthermore, enhancement of the insecticidal activity of Bt toxin alone, or a complete Bt formulation, by ACEV spindles is accompanied by PM disruption (Mitsuhashi et al. 2014a). Therefore, any microbes or materials that are unable to pass through the PM are likely to be able to reach the midgut cells after the PM is disrupted by fusolins (Fig. 7.2).

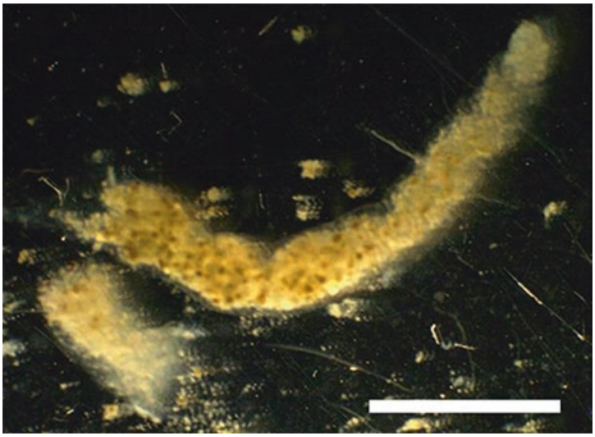


Fig. 7.2 Peritrophic matrix (PM) of *Bombyx mori* larvae. Reproduced from Mitsuhashi (2013). *Upper*, control PM. *Lower*, disrupted PM by action of spindles. Bar indicates 5 mm

7.8 Mechanism of PM Disruption

By assaying the activity of various ACEV fusolin mutants in a bioassay system that included BmNPV and *B. mori* larvae, our group identified the regions of fusolin that are involved in the enhancement of viral infectivity (Takemoto et al. 2008; Fig. 7.3). We found that the N-terminal region (ca. 250 aa) containing a signal peptide is essential for activity and N-glycosylation is important; the rest of the fusolin molecule is non-essential. We also found that the N-terminal region binds to chitin in vitro and that glycosylation is important for chitin binding (Takemoto et al. 2008). This essential region contains regions constituting a chitin-binding domain that are found in fusolins and proteins produced by many bacteria but very few eukaryotes. Indeed, *Spodoptera litura* NPV fusolin binds chitin in vitro (Li et al. 2003). The non-essential region is easily eliminated by serine protease(s) in insect digestive juice, whereas the essential region is more stable, and glycosylation is required for its stability (Takemoto et al. 2008).

Our group observed that a certain degree of disruption of the PM occurred immediately after the feeding of spindles to the larvae was completed and it was heavily disrupted several hours after the observation (our unpublished data; Mitsuhashi and Miyamoto 2003). However, the PM regenerated (except for its most posterior region) the next day (our unpublished data; Mitsuhashi and Miyamoto 2003).

Recently, the atomic structures of the fusolins and spindles of three EVs were determined by X-ray microcrystallography; for these studies, the in vivo spindles of *Melolontha melolontha* EV (genus *Alphaentomopoxvirus*), ACEV, and an EV from *Wiseana* spp. (genus *Betaentomopoxvirus*) that were purified from insects were used (Chiu et al. 2015) (Fig. 7.4). The structures were very similar to each other, and fusolins were found to be members of the auxiliary activity (AA) 10 family of lytic polysaccharide monooxygenases (LPMOs) on the basis of the features of the structures (Levasseur et al. 2013). Therefore it was elucidated that fusolin acts as a lytic chitin monooxygenase on the chitin of the PM, thereby disrupting it. AA10 LPMOs specifically hydrolyse crystalline polysaccharides (Aachmann et al. 2012), and it is speculated that more than 2000 organisms, including viruses, produce AA10 LPMOs (The Carbohydrate-Active enZYmes Database).

The N-terminal histidine (His1) of mature fusolin and a histidine located about 145 aa from the N-terminus form a histidine brace, a characteristic feature of LPMOs

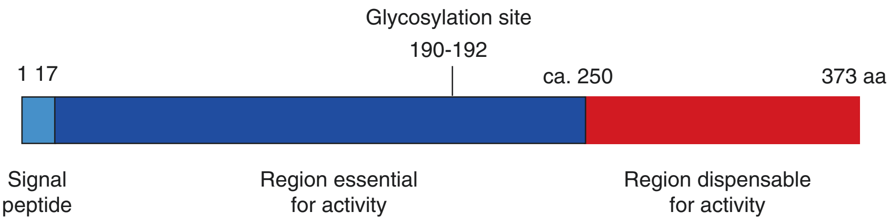


Fig. 7.3 Functional regions of *Anomala cuprea* EV fusolin. Functional regions were determined by using fusolin mutants. The region essential for activity can bind chitin; glycosylation enhances activity (*N*-glycosylation site; aa190–192). The region dispensable for activity is easily digested by insect midgut juice. aa, amino acid

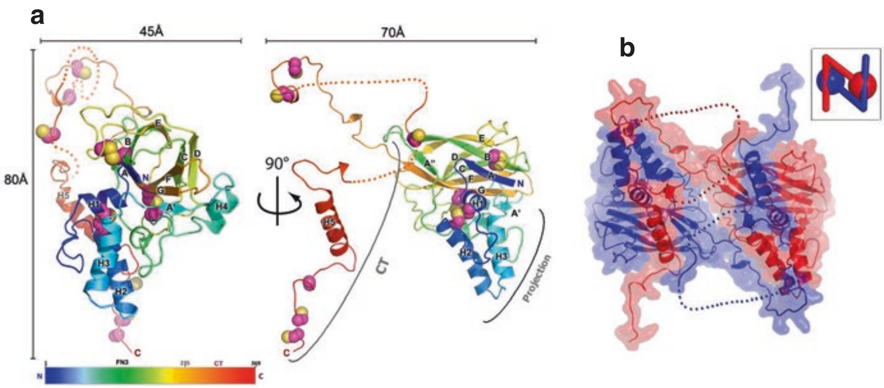


Fig. 7.4 The 3D structure of fusolin from *Melolontha melolontha* EV. Reproduced from Chiu et al. (2015). (**a**) Structure is represented by a ribbon diagram coloured in a *blue-red gradient* from the N- to the C-terminus. Cysteine residues are shown as spheres. (**b**) Fusolin forms a domain-swapped dimer shown as a ribbon diagram in a semi-transparent molecular surface. Inset: schematic representation of the dimer

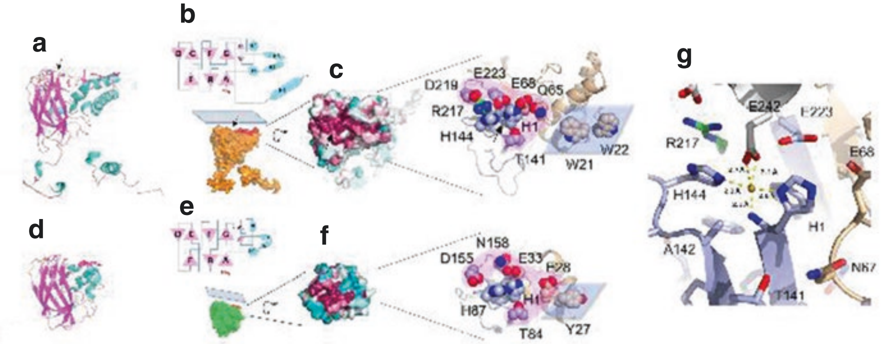


Fig. 7.5 Lytic chitin monooxygenase structures in fusolin of *Melolontha melolontha* EV. Reproduced from Chiu et al. (2015). Fusolin of *Melolontha melolontha* EV is compared with CBP21 of *Serratia marcescens*. (**a**) Fusolin. D, CBP21. Topologies (**b**, **e**, *top*) with a conserved flat platform on the molecular surface (**b**, **e**, *bottom*). Mapping of sequence variability onto the molecular surface revealed that the flat platform was highly conserved (**c**, **f**, *left*). The *blue-white-red gradient* represents low-to-high sequence conservation in 27 fusolins (**c**) and 400 CBP21-like proteins (**f**) (see Chiu et al. 2015). The two LPMO active sites are shown (**c**, **f**, *right*). Side chains of residues are shown as spheres. The projection is in *brown* and Fn3 domains are in *light blue*; Arg_{217} is in *green*, the polar rim in *pink* and the flat platform in *blue*. (**g**) Metal-binding site in *Melolontha melolontha* EV spindle. Colours are as in (**c**). A neighbouring molecule capping the site is shown in *white*. Although this site is occupied only by a water molecule in purified *Melolontha melolontha* EV (see Chiu et al. 2015), it can accommodate a Cu^{2+} after incubation in $CuSO_4$ solution (shown as a *brown sphere*). The metal-binding site is also indicated by *black arrows* in (**a**, **b**, **c**)

that consists of a surface-exposed metal-binding site located between the N-terminal amine of the strictly conserved His1 and the two imidazole side chains (Chiu et al. 2015) (Fig. 7.5). Another prominent feature of LMPOs is a di-tryptophan motif positioned to bind the planar surface of crystalline chitin (Chiu et al. 2015) (Fig. 7.5).

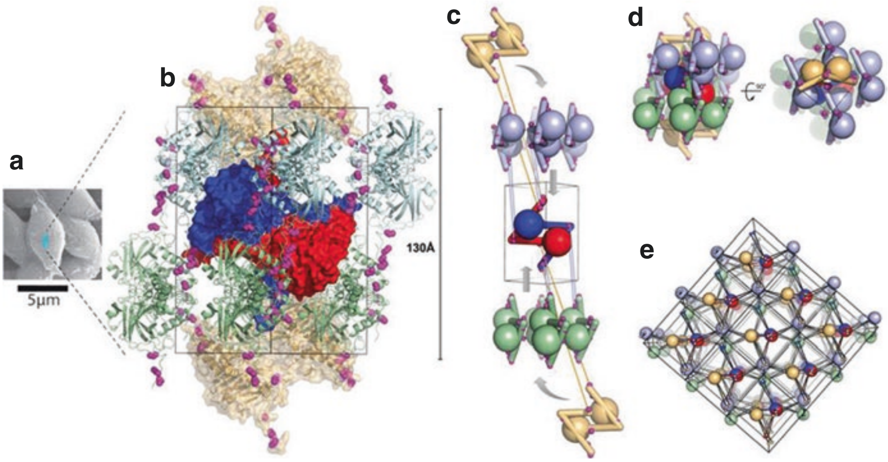


Fig. 7.6 Structures of the unit cell of spindle of *Melolontha melolontha* EV. Spindles are crystalline polymers of fusolin dimers cross-linked by a 3D network of disulphide bonds. The figures were cited from Chiu et al. (2015). (**a**) Scanning electron micrograph of spindles. A *blue box* represents a spindle unit cell (not to scale).(**b**) A unit cell viewed along a twofold crystallographic axis. Cysteines involved in inter-dimer cross-links are shown as *magenta spheres*. The central dimer is represented as a *blue-red* molecular surface; two crowns of four dimers are shown in *green* and *cyan*. The two capping dimers are shown in *brown* as a semi-transparent molecular surface. (**c**) Unit cell assembly. Inter-dimer disulphide bonds that involve the central dimer are indicated by links between the interacting cysteines. (**d**) The full unit cell is shown in the same orientation as in (**c**) and in an orthogonal view. (**e**) Representation of 18 unit cells with each dimer shown as a large sphere and cysteines as small spheres. Inter-sphere links highlight the 3D network of covalent bonds cross-linking spindles

Spindles are single crystals that diffract to high resolution (Chiu et al. 2015), although they are generally described as paracrystalline. In spindles, fusolin forms a dimer in which the disulphide bonds of the C-terminal extension mediate LPMO domain swapping within dimers (Chiu et al. 2015; Fig. 7.4). Spindles are formed by an intricate assembly of dimeric building blocks. The structure of a spindle unit cell is as follows. Fusolin dimers assemble to form two crowns of four dimers that encase a central dimer (Chiu et al. 2015; Fig. 7.6). Each crown is capped by a dimer from neighbouring cells that projects its C-terminal extensions through the central opening of the crown towards the central dimer. The central dimer is connected to both capping dimers and two dimers of each crown by disulphide bonds between the respective C-terminal extensions (Chiu et al. 2015; Fig. 7.6).

Spindles are inactive because the metal-binding site of fusolin is capped by an amino acid and neighbouring molecules occlude the planar platform containing the active site (Chiu et al. 2015). However, fusolin is released from the spindles by the combined action of the alkaline pH of midgut juice and degradation by midgut serine proteases (Takemoto et al. 2008; Chiu et al. 2015), and thus the active site is exposed. In addition to the intermolecular disulphide bonds stabilising spindles, the C-terminal region contains an unusually high proportion of tyrosines, which are located strategically at the dimer interface within helix H5 and around inter-dimer crystal contacts next to the stabilising disulphide bonds (Chiu et al. 2015). Thus,

dissolution may be facilitated by concerted deprotonation of the hydroxyl groups of tyrosines, introducing destabilising buried charges, and the weakening of disulphide bonds in the alkaline midgut (Chiu et al. 2015). A similar mechanism has been proposed for the polyhedra of NPVs and cypoviruses that infect insects, where tyrosine clusters are located close to molecular arms, which are analogous to the C-terminal region of fusolin (Chiu et al. 2012).

The protein CBP21 in the Gram-negative bacterium *Serratia marcescens*, which is sometimes virulent to insects, harbours a domain with chitinase activity and an AA10 LPMO domain, and it degrades chitin by the cooperative action of both domains (Vaaje-Kolstad et al. 2010). It is possible that fusolin also has chitinase activity against crystalline chitin.

The role of LPMOs in viruses differs from the primary role of many bacterial LPMOs, which is the degradation of polysaccharides as sources of nutrients. However, several proteins containing LPMO domains have also been associated with virulence in bacteria (Frederiksen et al. 2013), and it will be interesting to see whether bacterial and viral LPMOs affect the virulence of entomopathogenic microbes through similar mechanisms.

The enzymatic activity of LPMOs seems to be weak, and it was discovered only recently for CBP21 (Vaaje-Kolstad et al. 2010). However, the degree of disruption of the PM in *B. mori* larvae by the administration of ACEV spindles is drastic (Mitsuhashi and Miyamoto 2003), and thus a cofactor that boosts activity of fusolin is likely to be present. PM disruption by fusolin may include two steps. First, orally taken fusolin binds to the PM, oxidises its chitin fibrils and changes PM conformation; conformational changes induced in polysaccharides by AA10 proteins have been reported (Din et al. 1991, 1994). Second, these conformational changes allow digestive protease(s), which are unable to approach PM proteins before the first step, to access and digest them. Then, PM disruption is accelerated synergistically by fusolin and proteases owing to the increasing exposure of chitin fibrils and PM proteins.

Conclusion

Recent progress in research on fusolin has expanded its practical applicability. Strengthening of Bt insecticidal activity (shortening survival times and increasing the mortality rates of treated pests) remains desirable when such activity is insufficient. Recent research strongly suggests that fusolin spindles may be useful as a synergistic agent for Bt formulations and that expression of the fusolin gene in crops may allow us to reduce the amounts of microbial insecticides sprayed. Furthermore co-expressing fusolin with Bt toxin in crops may increase their resistance to insects (Liu et al. 2011; Mitsuhashi et al. 2014a).

A characteristic feature of fusolin crystals is their stabilisation by a 3D network of disulphide bonds that results in a fully cross-linked matrix (Chiu et al. 2015). This unique mechanism of fusolin stabilisation provides novel information to develop more stable LPMOs, which are important for boosting biofuel production from abundant recalcitrant biomass such as chitin and cellulose (Vaaje-Kolstad et al. 2010; Gelfand et al. 2013).

The following important problems remain to be resolved before fusolin can be practically used as a synergistic agent. Fusolin must be mass-produced at low cost. To this end, the use of a yeast expression system is promising. Unlike the expensive baculovirus expression system and the *E. coli* expression system, which does not allow glycosylation, the yeast system is cheap and allows glycosylation, which is important for high activity of fusolin.

It would also be important to improve the activity of fusolin by modifying the LPMO domain, the length of the C-terminal region, the number of cysteines and glycosylation.

ACEV spindles are not easily dissolved in the digestive systems of lepidopteran pest insects—especially of those that are not ACEV hosts (our unpublished data). Therefore, the development of methods (such as fusolin engineering) to facilitate the dissolution of ACEV spindles in the digestive system is necessary for their efficient use.

References

Aachmann FL, Sørlie M, Skjå-Bræk G, Eijsink VGH, Vaaje-Kolstad G (2012) NMR structure of a lytic polysaccharide monooxygenase provides insight into copper binding, protein dynamics, and substrate interactions. Proc Natl Acad Sci U S A 109:18779–18784

Adams JR, Wilcox TA (1968) Histopathology of the almond moth, *Cadra cautella*, infected with a nuclear-polyhedrosis virus. J Invertebr Pathol 12:269–274

Afonso CL, Tulman ER, Lu Z, Oma E, Kutish GF, Rock DL (1999) The genome of *Melanoplus sanguinipes* entomopoxvirus. J Virol 73:533–552

Bawden AL, Glassberg KJ, Diggans J, Shaw R, Farmerie W, Moyer RW (2000) Complete genomic sequence of the *Amsacta moorei* entomopoxvirus: analysis and comparison with other poxviruses. Virology 274:120–139

Chakraborty M, Narayanan K, Sivaprakash MK (2004) *In vivo* enhancement of nucleopolyhedrovirus of oriental armyworm, *Mythimna separata* using spindles from *Helicoverpa armigera* entomopoxvirus. Indian J Exp Biol 42:121–123

Chakraborty M, Narayanan K, Suryanarayana VVS, Sivaprakash MK (2005) Enhancement of nucleopolyhedrovirus of oriental armyworm, *Mythimna separata* (Lepidoptera: Noctuidae) using diamond shaped inclusion bodies of *Galleria mellonella* NPV (Lepidoptera: Pyralidae). Entomon 30:343–346

Chiu E, Coulibaly F, Metcalf P (2012) Insect virus polyhedra, infectious protein crystals that contain virus particles. Curr Opin Struct Biol 22:234–240

Chiu E, Hijnen M, Bunker R, Boudes M, Rajendran C, Aizel K, Olieric V, Schulze-Briese C, Mitsuhashi W, Young V, Ward VK, Bergoin M, Metcalf P, Coulibaly F (2015) Structural basis for the enhancement of virulence by viral spindles and their *in vivo* crystallization. Proc Natl Acad Sci U S A 112:3973–3978

Derksen ACG, Granados RR (1988) Alteration of a lepidopteran peritrophic membrane by baculoviruses and enhancement of viral infectivity. Virology 167:242–250

Din N, Gilkes NR, Tekant B, Miller RC, Warren AJ, Kilburn DG (1991) Non-hydrolytic disruption of cellulose fibres by the binding domain of a bacterial cellulose. Nat Biotechnol 9:1096–1099

Din N, Damude HG, Gilkes NR, Miller RC Jr, Warren RAJ, Kilburn DG (1994) C_1-C_x revisited: intramolecular synergism in a cellulase. Proc Natl Acad Sci U S A 91:11383–11387

Frederiksen RF, Paspaliari DK, Larsen T, Storgaard BG, Larsen MH, Ingmer H, Palcic MM, Leisner JJ (2013) Bacterial chitinases and chitin-binding proteins as virulence factors. Microbiology 159:833–847

Furuta Y, Mitsuhashi W, Kobayashi J, Hayasaka S, Imanishi S, Chinzei Y, Sato M (2001) Peroral infectivity of non-occluded viruses of *Bombyx mori* nucleopolyhedrovirus and polyhedrin-negative recombinant baculoviruses to silkworm larvae is drastically enhanced when administered with *Anomala cuprea* entomopoxvirus spindles. J Gen Virol 82:307–312

Gelfand I, Sahajpal R, Zhang X, Izaurralde RC, Gross KL, Robertson GP (2013) Sustainable bioenergy production from marginal lands in the US Midwest. Nature 493:514–517

Goto C (1990) Enhancement of a nuclear polyhedrosis virus (NPV) infection by a granulosis virus (GV) isolated from the spotted cutworm, *Xestia c-nigrum* L. (Lepidoptera: Noctuidae). Appl Entomol Zool 25:135–137

Gross CH, Wolgamot GM, Russell RLQ, Pearson MN, Rohmann GF (1993) A 37-kilodalton glycoprotein from a baculovirus of *Orgyia pseudotsugata* is localized to cytoplasmic inclusion bodies. J Virol 67:469–475

Hayakawa T, Shitomi Y, Miyamoto K, Hori H (2004) GalNAc pretreatment inhibits trapping of *Bacillus thuringiensis* Cry1Ac on the peritrophic membrane of *Bombyx mori*. FEBS Lett 576:331–335

Hayakawa T, Xu J, Hukuhara T (1996) Cloning and sequencing of the gene for an enhancing factor from *Pseudaletia separata* entomopoxvirus. Gene 177:269–270

Huger AM, Krieg A (1968) On spindle-shaped cytoplasmic inclusions associated with a nuclear polyhedrosis of *Choristoneura murinana*. J Invertebr Pathol 12:461–462

Hukuhara T, Tamura K, Zhu Y, Abe H, Tanada Y (1987) Synergistic factor shows specificity in enhancing nuclear polyhedrosis virus infections. Appl Entomol Zool 22:235–236

Lai-Fook J, Dall DJ (2000) Spindle bodies of *Heliothis armigera* entomopoxvirus develop in structures associated with host cell endoplasmic reticulum. J Invertebr Pathol 75:183–192

Levasseur A, Drula E, Lombard V, Coutinho PM, Henrissart B (2013) Expansion of the enzymatic repertoire of the CAZy database to integrate auxiliary redox enzymes. Biotechnol Biofuels 6:41

Li X, Barrett J, Pang A, Klose RJ, Krell PJ, Arif BM (2000) Characterization of an overexpressed spindle protein during a baculovirus infection. Virology 268:56–67

Li Z, Li C, Yang K, Wang L, Yin C, Gong Y, Pang Y (2003) Characterization of a chitin-binding protein GP37 of *Spodoptera litura* multicapsid nucleopolyhedrovirus. Virus Res 96:113–122

Liu X, Ma X, Lei C, Xiao Y, Zhang Z, Sun X (2011) Synergistic effects of *Cydia pomonella* granulovirus GP37 on the infectivity of nucleopolyhedroviruses and lethality of *Bacillus thuringiensis*. Arch Virol 156:1707–1715

Mitsuhashi W (2002) Further evidence that spindles of an entomopoxvirus enhance its infectivity in a host insect. J Invertebr Pathol 79:59–61

Mitsuhashi W (2009) Insect virus proteins involved in the peroral infectivity of the viruses and their potential practical application in pest control. In: Insect viruses: detection, characterization and roles. Nova Science Publishers, New York, pp 1–20

Mitsuhashi W (2013) Trends in studies on insect viral proteins involved in peroral infectivity of insect viruses. Sanshi-Konchu Biotechnol 82:99–108. (in Japanese)

Mitsuhashi W, Asano S, Miyamoto K, Wada S (2014a) Further research on the biological function of inclusion bodies of *Anomala cuprea* entomopoxvirus, with special reference to effect on the insecticidal activity of a *Bacillus thuringiensis* formulation. Pest Manag Sci 70:46–54

Mitsuhashi W, Miyamoto K, Wada S (2014b) The complete genome sequence of the *Alphaentomopoxvirus Anomala cuprea entomopoxvirus*, including its terminal hairpin loop sequences, suggests a potentially unique mode of apoptosis inhibition and mode of DNA replication. Virology 452–453:95–116

Mitsuhashi W, Furuta Y, Sato M (1998) The spindles of an entomopoxvirus of *Coleoptera* (*Anomala cuprea*) strongly enhance the infectivity of a nucleopolyhedrovirus in *Lepidoptera* (*Bombyx mori*). J Invertebr Pathol 71:186–188

Mitsuhashi W, Kawakita H, Murakami R, Takemoto Y, Saiki T, Miyamoto K, Wada S (2007) Spindles of an entomopoxvirus facilitate its infection of the host insect by disrupting the peritrophic membrane. J Virol 81:4235–4243

Mitsuhashi W, Miyamoto K (2003) Disintegration of the peritrophic membrane of silkworm larvae due to spindles of an entomopoxvirus. J Invertebr Pathol 82:34–40
Mitsuhashi W, Murakami R, Takemoto Y, Miyamoto K, Wada S (2008) Stability of the viral-enhancing ability of entomopoxvirus spindles exposed to various abiotic factors. Appl Entomol Zool 43:483–489
Mitsuhashi W, Sato M (2000) Enhanced infection of a nucleopolyhedrovirus in a lepidopteran pest (*Spilosoma imparilis*) by spindles of a coleopteran entomopoxvirus (EPV) (*Anomala cuprea* EPV). J For Res 5:285–287
Mitsuhashi W, Sato M, Hirai Y (2000) Involvement of spindles of an entomopoxvirus (EPV) in infectivity of the EPVs to their host insect. Arch Virol 145:1465–1471
Olszewski JA, Dall DJ (2002) Assessment of foreign protein production by recombinant *Heliothis* (*Helicoverpa*) *armigera* entomopoxviruses in *Spodoptera frugiperda* cells. J Gen Virol 83:451–461
Phanis CG, Miller DP, Cassar SC, Tristem M, Thiem SM, O'Reilly DR (1999) Identification and expression of two baculovirus *gp37* genes. J Gen Virol 80:1823–1831
Peng J, Zhong J, Granados RR (1999) A baculovirus enhancin alters the permeability of a mucosal midgut peritrophic matrix from lepidopteran larvae. J Insect Physiol 45:159–166
Salvador R, Ferrelli ML, Berretta MF, Mitsuhashi W, Biedma ME, Romanowski V, Sciocco-Cap A (2012) Analysis of EpapGV *gp37* gene reveals a close relationship between granulovirus and entomopoxvirus. Virus Genes 45:610–613
Takemoto Y, Mitsuhashi W, Murakami R, Konishi H, Miyamoto K (2008) The N-terminal region of an entomopoxvirus fusolin is essential for the enhancement of peroral infection, whereas the C-terminal region is eliminated in digestive juice. J Virol 82:12406–12415
Tanada Y (1959) Synergism between two viruses of the armyworm, *Pseudaletia unipuncta* (Haworth) (Lepidoptera, Noctuidae). J Invertebr Pathol 1:215–231
Thézé J, Takatsuka J, Nakai M, Arif B, Herniou EA (2015) Gene acquisition convergence between entomopoxviruses and baculoviruses. Viruses 7:1960–1974
Vaaje-Kolstad G, Westereng B, Horn SJ, Liu Z, Zhai H, Sørlie M, Eijsink VGH (2010) An oxidative enzyme boosting the enzymatic conversion of recalcitrant polysaccharides. Science 330:219–222
Vialard JE, Yuen L, Richardson CD (1990) Identification and characterization of a baculovirus occlusion body glycoprotein which resembles spheroidin, an entomopoxvirus protein. J Virol 64:5804–5811
Wang P, Granados RR (1997) An intestinal mucin is the target substrate for a baculovirus enhancin. Proc Natl Acad Sci U S A 94:6977–6982
Wang P, Granados RR (2000) Calcofluor disrupts the midgut defense system in insects. Insect Biochem Mol Biol 30:135–143
Wang P, Granados RR (2001) Molecular structure of the peritrophic membrane (PM): identification of potential PM target sites for insect control. Arch Insect Biochem Physiol 47:110–118
Wang P, Hammer DA, Granados RR (1994) Interaction of *Trichoplusia ni* granulosis virus-encoded enhancin with the midgut epithelium and peritrophic membrane of four lepidopteran insects. J Gen Virol 75:1961–1967
Wijonarko A, Hukuhara T (1998) Detection of a virus enhancing factor in the spheroid, spindle, and virion of an entomopoxvirus. J Invertebr Pathol 72:82–86
Xu J, Hukuhara T (1992) Enhanced infection of a nuclear polyhedrosis virus in larvae of the armyworm, *Pseudaletia separata*, by a factor in the spheroids of an entomopoxvirus. J Invertebr Pathol 60:259–264
Yaman M, Acar KF, Radek R (2015) A nucleopolyhedrovirus from the Mediterranean flour moth, *Ephestia kuehniella* (Lepidoptera: Pyralidae). Appl Entomol Zool 50:355–359

Antiviral Mechanism of Serine Protease in Various Insects

8

Jyoti Verma

Abstract

During the last two decades, insects have had the self-ability to develop a vigorous and potent immune system that contests a huge diversity of pathogens and lead them to grow into the most distinct and efficient organisms in the world. Immune reactions against pathogens are basically characterized by invasion of their cellular and humoral response. In the present era of challenging environmental conditions, there is an urgent need for the prevention and control of viral diseases. Serine proteases (SPs) are a vast group of proteolytic enzymes that play an enormous role in anatomical systems (cell signaling, defenses, and movement, etc.); thus, they are crucial to the antiviral mechanism (hemolymph coagulation, activation of antimicrobial peptide, and melanin synthesis). They participate in various biochemical and physiochemical pathways and act as catalysts that break down the peptide bond in the protein. SPs are vital to numerous microorganisms and contribute to several structural and biochemical concerns, including a conserved catalytic triad (Ser, Asp, and His) that enacts the fundamental principle for the classification of a protein. SPs have diverse functions and play a vital role in cellular differentiation, digestion, complement activation, the immune response, and hemostasis. Recently, immunological responses in many insects such as *Bombyx mori*, Drosophila, Anopheles, etc., are maintained by circulatory hemocytes and performed a significant role in innate immune system, namely, the synthesis of antimicrobial proteins, encapsulation, and phenoloxidase. Most of the antimicrobial proteins such as cecropins, attacins, lebocin, moricin, gloverins, lysozyme, defensins, hemolin, etc., are effectively engaged in defense reactions against invading pathogens. For antiviral mechanisms, molecular and cell target-based analysis are valuable studies for identifying the genome

J. Verma
Department of Biotechnology, Babasaheb Bhimrao Ambedker University, Lucknow, UP, India
e-mail: jyoti.verma873@gmail.com

D. Kumar, C. Gong (eds.), *Trends in Insect Molecular Biology and Biotechnology*, https://doi.org/10.1007/978-3-319-61343-7_8

and expression analysis of SPs, and their homologs in the silkworm, *Drosophila melanogaster*, *Apis mellifera*, and *Anopheles gambiae*, are generally considered to be model organisms for providing the relevant information regarding such biological functions. In this chapter, we devote our endeavors to the antiviral mechanism of SPs in various insects and critique the recent data on visualizing the role of antiviral pathways. Furthermore, the antiviral pathways may encounter the infectious virus towards the systemic and specific level.

8.1 Introduction

Insect resistance plays a relevant role in the synergism within the host–pathogen relationship, as a part of a survival strategy, along with physical impediments such as epithelial barriers, cuticle and peritrophic matrix, protease cascades leading to coagulation and melanization, and fundamental cellular responses (Choo et al. 2010; Feng et al. 2013; Wang et al. 2016; Lavin and Strand 2002; Lehane et al. 2004; Kotani et al. 1999). For many decades, the defense systems of insects, in contrast to various pathogens such as bacteria, fungus, and protozoa, have been well recognized, but articles on antiviral mechanism are scarce and surprising owing to a lack of understanding of the mechanism of virus invasion and host–virus response (Popham et al. 2004). In an earlier report, insect baculoviruses were accomplished in recombinant protein expression systems, but there are virtually no studies including the antiviral mechanisms against viruses. Thus, there is specific consideration of *Bombyx mori*, which is attracted to the area to regulate the gene and protein and to balance expression in genetically modified cell lines (Popham et al. 2004). Serine proteases (SPs) act as hydrolytic enzymes that are incorporated into the conserved catalogue of triad residue (His, Ser, and Asp) and are routinely integrated as idle zymogen with propeptide, which must be evacuated for their activation (Ross et al. 2003). Most studies have delineated that insect SPs perform a fundamental aspect of dietary protein digestion (Herrero et al. 2005; Soares et al. 2011), molting (Wei et al. 2007; Liu et al. 2009; He et al. 2009), metamorphosis (Danielli et al. 2000; Kaji et al. 2009), and the immune response (An et al. 2009), whereas SP homologs(SPHs) are identical to SPs in amino acid sequences; nevertheless, there is a scarcity in amidase activity because of mutation (Romualdi et al. 2003). In various reports, *Anopheles gambiae*, *Drosophila melanogaster* and *Bombyx mori*, and *Bombus ignitus* contain 305, 206, and 143 SP or SPH genes, 1,720 bp respectively (Choi et al. 2006; Romualdi et al. 2003; Zdobnov et al. 2002; Zhao et al. 2010) and are categorized into different families (Table 8.1). Insects have the ability to develop mechanisms that resist various pathogens, including viruses (Qin et al. 2012). Choo et al. (2010) investigated the bee venom SPs (Bi-VSPs), which promote the arthropods prophenoloxidase (proPO)-activating factors (PPAFs) via a melanization process and illustrated fibrin(ogen)

Table 8.1 List of gene number of serine proteases and serine protease homolog gene in different insects (adopted from Zhao et al. 2010)

Family	*D. melanogaster*	*A. gambiae*	*A. mellifera*	*B. mori*
SP_fam1	65	49	28	24
SP_fam2	12	37	4	9
SP_fam3	18	22	2	6
SP_fam4	17	19	4	5
SP_fam5	2	29	–	4
SP_fam6	9	9	1	2
SP_fam7	3	4	2	1
SP_fam8	–	1	0	5
SP_fam9	1	1	1	2
SP_fam10	1	1	1	1
SP_fam11	1	1	1	1
SP_fam12	–	49	28	15
SP_fam13	–	37	4	7
SP_fam14	–	22	2	6
SP_fam15	–	19	4	5
SP_fam16	–	29	–	3
SP_fam17	–	9	1	3

activity by eradication of insects. Furthermore, they reported that SP constitutes of multifunctional enzymes that stimulate prothrombin without any deviation, degenerating the fibrinogen into fibrin-like products. In this chapter, we emphasize the immune response against viral infection and how to consider the aspects of SP via different mechanisms.

For 300 billion years, *Bombyx mori* has represented great economic value from an industrial and a pharmaceutical and medicinal point of view. In the current scenario, there are major economic resources found in various countries, e.g., India, China, Vietnam, and Thailand. (Qin et al. 2012) and are used as bioreactors to produce clinically significant biomolecules such as human granulocyte macrophage colony-stimulating factor (Xia et al. 2004; Chen et al. 2006). Certain immune mechanisms also exist in *Bombyx mori* and they are susceptible to *B. mori* nuclear polyhedrosis virus virus (BmNPV) infection (Qin et al. 2012). Various researchers have also reported that BmNPV acts as a primary pathogen of the domestic silkworm and induces large commercial losses (Cheng et al. 2014; Miao et al. 2005). Most silkworm strains are highly sensitive to BmNPV, but rarely show high resistance to BmNPV (Bao et al. 2009). Recently, some studies on insect resistance to BmNPV, i.e., *Bombyx mori* SP-2, lipase-1, and alkaline trypsin protein purified from the digestive juice of *B. mori* larvae, have demonstrated strong antiviral activity to BmNPV in vitro (Nakazawa et al. 2004; Ponnuvel et al. 2003; Ponnuvel et al. 2012). Here, we attempt to consolidate the antiviral mechanism of SP in different insects (Figs. 8.1 and 8.2).

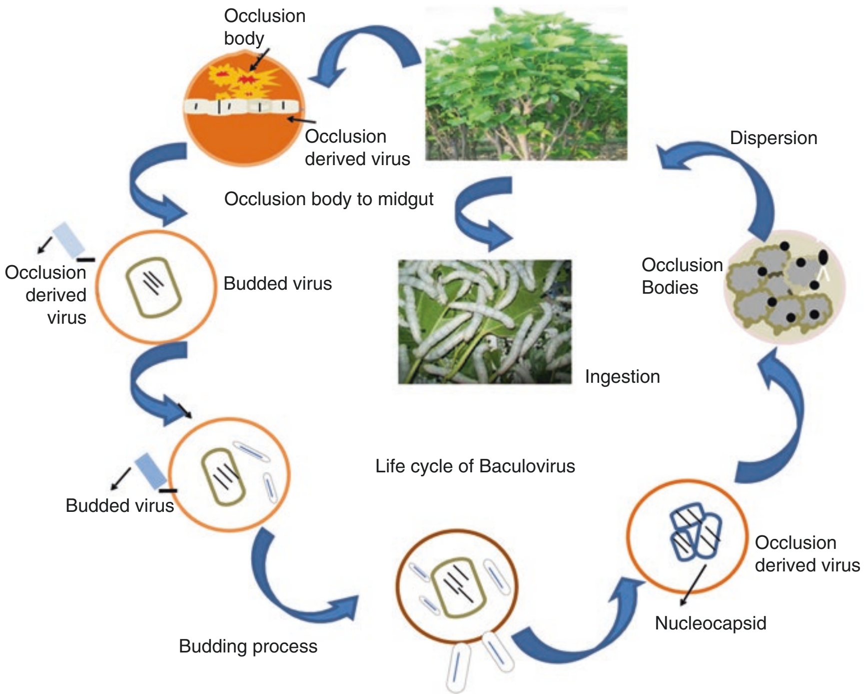


Fig. 8.1 Life cycle of baculovirus in an antiviral mechanism

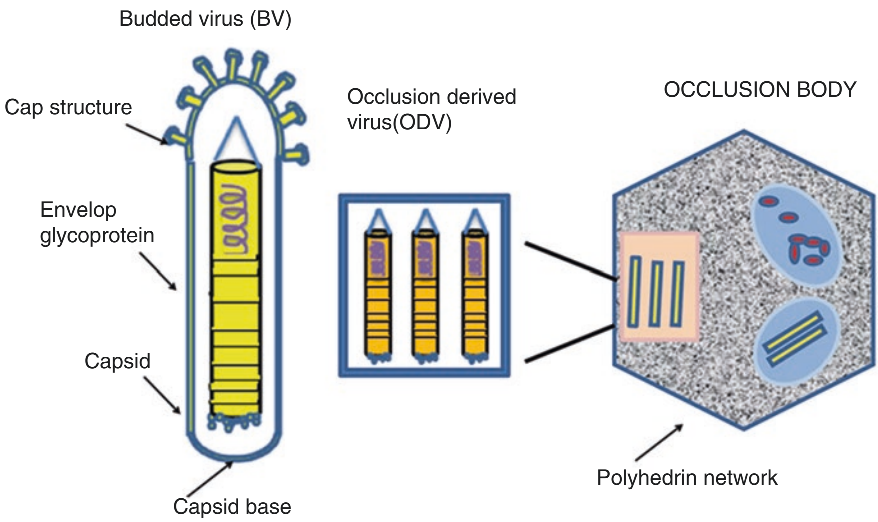


Fig. 8.2 Detailed structure of budded virus (BV) and occlusion-derived virus (ODV)

8.2 What Is Serine Protease?

Proteases are the largest group of enzymes, ubiquitous in nature, that hydrolyze proteins by adjoining water across peptide bonds (Saleem et al. 2012) and catalyze peptide construction in an organic solvent with a low water content (Soares et al. 2011). According to Verma et al. (2011) proteases are subdivided into diverse groups based on their catalytic activity with reference to reaction medium and can be identified as acidic, alkaline, neutral, and in active site groups. Proteases are also classified according to three major criteria: (1) the type of reaction catalyzed, (2) the chemical nature of the catalytic site, and (3) the evolutionary relationship with regard to structure (Barett 1994). Proteases are also categorized into exo- and endopeptidases depending on their action at or away from termini respectively and subdivided based on the nature of their functional active site groups, i.e., SPs, aspartic proteases, cysteine proteases, and metalloproteases (Hartley 1960). Of these functions, SPs act as mediators among the immune systems of different insects and determine the defense mechanisms of various pathogens via antimicrobial peptide synthesis, hemolymph coagulation, and melanization of pathogen surfaces (Gorman and Paskewitz 2001). Previously, a protein was demonstrated to have a significant antiviral activity against BmNPV from the digestive juice of *Bombyx mori* and was termed *B. mori* SP (BMSP-2) (Nakazawa et al. 2004). According to Kotani et al. (1999), BmSp-2 exhibited 94% amino acid sequence identity with SP. Recently, Lin et al. (2017) reported that SP inhibitors (SPIs) were present in all living animals and performed a vital role in development, digestion, and innate immunity. They studied the genome-wide characterization and expression profiling of the *SPI* gene in *Plutella xylostella* and noted that the *SPI* gene was categorized into serpins, canonical inhibitors, and alpha-2-macroglobulins. Of these, serpins demonstrated an association with the regulation of innate immunity of insects, whereas according Zhao et al. (2010), the upregulation and downregulation of different SP inhibitor genes may be participating in the combat with pathogenic microorganisms such as *Escherichia coli*, *Bacillus bombysepticus*, *Beauveria bassiana*, or BmNPV.

8.3 Role of Serine Protease in National and International Industries

In earlier studies, a diverse group of proteins and SPs played a very significant role in insect immunity for the mechanism of antiviral infection and were referred to as "serpocidins" (Kim et al. 2009). Of the various proteases, SPs have a zymogen-like structure that comprises the catalytic domain of the C-terminal and the regulatory domain of the N-terminal, are generally activated by His/Asp/Ser from the catalytic site, and participate in the regulatory mechanism of melanization, coagulation, and antimicrobial peptide production, through protease cascades (Kim et al. 2009; Gabay 1994; Gorman and Paskewitz 2001). In addition, SP homologs possess the catalytic triad and play a vital role in the antibacterial mechanism of human azurocidin (Gabay and Almeid 1993) and horseshoe crab factor D (Iwanaga 2002); cell

adhesion, *Drosophila* masquerade (Murugasu-Oei et al. 1995); *Pacifastacus* masquerade-like protein (Huang et al. 2000); prophenoloxidase activation, *Holotrichia* masquerade-like protein (Kwon et al. 2000); and immune function, *Anopheles* SP15 (Dimopoulos et al. 1997) and human hepatocyte growth factor (Nakamura et al. 1989).

In several regions of the world, arbovirus (arthropod-borne viruses) has been shown to be a risk to animal and human health and there is scanty knowledge about arbovirus infection (Fragkoudis et al. 2009). Previously, vertebrate immunity to viral infection could be regulated by the JAK/STAT signaling pathway, virus interference, and the virus nucleic acid sensor pathway (Randall and Goodbourn 2008). However, during the last few years, there has been a vast increase in knowledge on mosquito genetics and immunity-related genes, mainly through the *Anopheles gambiae* and *Aedes aegypti* sequencing projects (Christophides et al. 2002; Holt et al. 2002). Recently, Fragkoudis et al. (2009) reported that most arboviruses are RNA virus families such as Bunyaviridae, Togaviridae, and Flaviviridae, although the bluetongue virus is from the double-stranded RNA (dsRNA) family Reoviridae, an arbovirus family of great veterinary importance. The immune pathway involved in arbovirus–mosquito interactions has largely relied on genomic studies to identify differentially regulated genes (Ross et al. 2003; Wang et al. 2008). In the midguts of *Anopheles gambiae* and *Aedes aegypti* infected with SINV, upregulation of the Toll pathways is followed by activation of JNK signaling and is probably preceded by IMD activation (both pathways are linked in *D. melanogaster*) (Ross et al. 2003). In addition, other immune molecules such as SPs are upregulated and play an important role in innate immunity (Lemaitre and Hoffmann 2007); however, their role in the response to viral infections remains unclear. In the case of another alphavirus, SFV, if activated before infection, not Toll- but Gram-negative-mediated signaling (JAK/STAT or IMD/JNK) can inhibit virus replication in mosquito cell cultures (Fragkoudis et al. 2008; Jiang et al. 2009).

Jiang et al. (2009) and Gorman et al. (2000b) reported that SP plays a crucial role in an insect immune pathway for the synthesis of melanin, but in the process of melanization it acts as activator in mosquitoes and *Drosophila*-like insects. In this regard, the Sp22D protein sequence is determined by quantitative northern blot analysis and in-situ hybridization. Gorman et al. (2000a) also reported that Sp22D codes for a 1322 amino acid polypeptide with a complex domain organization in *Anopheles gambiae*. In addition to the SP catalytic domain, Sp22D contains two putative chitin-binding domains, a mucin-like domain, two low-density lipoprotein receptor class A domains, and two scavenger receptor cysteine-rich domains, and participates in translational upregulation. A few years earlier, Choo et al. (2007) described an SP characterized based on cDNA cloning, expression, and enzyme activity in the midgut of the *Bombus ignitus* and consisting of four introns and five exons coding for 250 amino acid residues. Wang et al. (2008) also suggested that infection of mosquitoes with recombinant arboviruses expressing activators or inhibitors of apoptosis might be used in the regulation of antiviral mechanisms.

Recently, the SP homolog SPH-3 (an insect non-clip domain-containing SPH) played a central role in insect immunity in *Manduca sexta* infection, with a virulent,

insect-specific, Gram-negative bacterium *Photorhabdus luminescens* (Felfoldi et al. 2011). Felfoldi et al. (2011) reported that RNA interference suppression of bacteria induced SPH-3 synthesis severely compromises the insect's ability to defend itself against infection by preventing the transcription of multiple antimicrobial effector genes, but, surprisingly, not the transcription of immune recognition genes. After that, gene encoding prophenoloxidase was performed by upregulation and the activity of the phenoloxidase enzyme are among the antimicrobial responses that are severely attenuated on SPH-3 knockdown, which concluded that SPH-3 regulates the genes encoding pattern in signaling pathways and controls the infection. As a nonfunctional serine proteinase homolog, SPH-3 cannot be an enzymatically active proteolytic component in a signaling cascade. However, the signaling pathway akin to the Toll family receptor in *Drosophila*, which may play a key adaptor in signal-mediating upstream toward the receptor. This finding indicates that study of viral and microbial infections is important for conferring the immune response in different insects, such as *Bombyx mori*, *Drosophila*, *Anopheles*, *Manduca sexta*, along with its prevention technology.

8.4 Serine Protease Mechanism and Specificity

The process of catalysis and specificity are not quietly inhibited by a scant residue, nevertheless effects on the whole protein frameworks disciplined via the rationing of the hydrogen bond charges, as it may be linking of domain drift to the chemical conversion (Hedstrom 2002). The specificity of SP is basically deliberated the physiography of the substrate binding sites which can adjoin the catalytic site of cleft (Polgar 2005). However, in an earlier report, SP specificity has produced a lot of information regarding biological function and the establishment of efforts (Perona and Craik 1995). Of the industrial enzymes, about 75% of microbial proteas belong to SPs and serve the nucleophilic Ser residue at their functioning site (Rao et al. 1998). SPs are classified as the catalytic triad with the presence of Asp, His, and Ser, and these are designated as catalytic machinery, which is subdivided into four separate groups. In an earlier report, these four groups of SPs are described as chymotrypsin, subtilisin, carboxypeptidase Y, and Clp protease (Rawlings and Barrett 2000).

8.5 Silkworm: Mode of Infection and Mechanism of Serine Protease as an Antiviral Factor

Feng et al. (2013) reported that insects get participated in different ways to defend themselves against different pathogen such as fungi, bacteria, nematodes but scanty knowledge about the insect immune response against viruses. Among them, one of the most important viruses is *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV), which spreads almost 30 lepidopteron species, whereas baculoviruses have a very narrow host range that contains a circular double-stranded genome ranging from 80 to 180 kbp and moderated in the form of larvae of *Bombyx mori* (An et al. 2009; Rahman and Gopinathan 2004). The life cycle of *Bombyx mori* contains two

specific forms of virus, occlusion-derived virus (ODV) and the budded virus (BV). Both forms play a different role in the interim pathogenesis in Fig. 8.1 (Katsuma et al. 2006). Antiviral immunity in other insects is well known, but there is not much more information on the silkworm, *B. mori*. In addition, other factors such as hemolin, receptors in midgut epithelial cells, phenoloxidase, and apoptosis also played a significant role in the antiviral mechanism (Ponnuvel et al. 2012). Recently, a protein RNAse III (dicer) was identified that showed the antiviral mechanism against infectious flu virus (IFV) (Ponnuvel et al. 2008; Ponnuvel et al. 2012). Nakazawa et al. (2004) reported that *Bombyx mori* possesses the two forms of virus particles, BmNPV and AcMNPV, which showed that they are phenotypically different, but genetically identical, and complied with their life cycle at the time of pathogenesis. The two forms play different roles. During this process, infection begins and the ODV fuses with the microvillar membrane (Monsma et al. 1996). This virus particle is enclosed with proteinaceous occlusions, which are discharged into the midgut of the larvae with the combination of alkaline gut pH and protease (Engelhard and Volkman 1995). The cell is infected and produces many primary single nucleocapsids through the basal membrane. This BV gained an envelope studded with glycoprotein GP-64, which infected the neighboring host and tissues (Monsma et al. 1996). The detailed structure of budded virus (BV) and occlusion-derived virus (ODV) is summarized in Fig. 8.2.

Various researchers reported that SP played a significant role in antiviral activity against BmNPV, which is designated as BmSP-2 and analyses their gene expression in the midgut of *Bombyx mori* (Nakazawa et al. 2004; Ponnuvel et al. 2008), whereas Zhao et al. (2010) presumed that potential SPI genes based on the genome sequences of the silkworm are susceptible to the antiviral mechanism. He reported that these SPI genes may be responsible for defenses to pathogenic microorganisms through microarray and qRT-PCR assay. This report highlights the upregulation and downregulation of several SPI genes subsequently infected by *Escherichia coli*, *Bacillus bombysepticus*, *Beauveria bassiana*, or BmNPV. Recently, Liu et al. (2016) investigated the roles of SP for antiviral mechanisms and identified SP gene BmSP36, which has a 292-residue protein and is cloned. Liu et al. (2016) also defined that the BmSP36 consists of an intact catalytic triad (H57, D102, and S195) and a conserved substrate binding site (G189, H216, and G226), which is responsible for chymotrypsin-like specificity. According to their reports, BmSP36 plays a significant role in the midgut of *B. mori* and they analyzed the transcriptional and translational expression using western blotting, immunofluorescence, and liquid chromatography-tandem mass spectrometry assay.

8.6 Anopheles: Mode of Infection and Mechanism of Serine Protease as an Antiviral Factor

Anopheles serves as an obligate intermediate parasite, which is harmful to humans. In a susceptible mode during blood feeding in the midgut, it encounters the multiple rhythms of reproduction and development before ascending to the salivary glands (Gorman et al. 2000b). Various numbers of parasites complete

their life cycle in susceptible mosquitoes, which is interrupted by various mechanisms and only a very small proportion succeeds (Beier 1998; Gorman et al. 2000b). Richman et al. (1996) also reported that the antimicrobial peptides function as defensins and cecropins against parasitic infection in the mosquito. Ashida and Brey (1997) reported that melanotic encapsulation and the production of antimicrobial peptides are types of innate, humoral immune responses regulated by SPs, mediated the cleavage of prophenoloxidase (proPO), and instigated the construction of reactive quinones, which cross-link to form melanin, whereas melanotic encapsulation generated the refractory lines of mosquitoes in the process of melanization and killed the parasites completely (Gorman et al. 2000b). For many decades, mosquito-borne arboviral disease (such as dengue), was responsible for an estimated 300 million infections annually (Bhatt et al. 2013). The replication cycle of the dengue virus is carried in the Ades mosquito for 7–14 days and can fluctuate with regard to virus performance and temperature (Alto and Bettinardi 2013). In an earlier study, Souza-Neto et al. (2009) reported that the Janus kinase/signal transducer and activator of the transcription (JAK/STAT) pathway regulated a conserved immune signaling pathway for the development of antiviral mechanisms in both mammals and insects, and previously hypothesized that the JAK/STAT pathway regimented dengue infection in *A. aegypti*. Recently, Jupatanakul et al. (2017) investigated the JAK/STAT pathway, which conserved the immune signaling pathway and regulated developmental processes and antiviral immunity in both mammals and insects with viral infection. In this method, activation of the JAK/STAT pathway through RNAi-mediated gene silencing of a protease inhibitor such as SP-activated STAT (PIAS) renders mosquitoes more resistant to DENV infection of the midgut, whereas silencing of the receptor Dome or the Janus kinase Hop renders the mosquitoes more susceptible to DENV infection (Souza-Neto et al. 2009). SPs activate the signaling pathways that accelerate the transcription of the genes and five new SP (Sp14A, Sp14D1, Sp14D2, Sp18D, and Sp22D) cDNAs from the hemolymph of the malaria vector (*Anopheles gambiae*) were identified, which significantly enhanced the melanotic encapsulation of plasmodium and antiviral immunity (Gorman et al. 2000b). Thus, there are urgent needs from the last few decades regarding dengue virus (DENV: *Flavivirus*), which poses a significant risk to human health , and the scarcity of the drugs required for the prevention and control of dengue disease.

8.7 *Drosophila*: Mode of Infection and Mechanism of Serine Protease as an Antiviral Factor

Recently, the mode of infection and mechanism of antiviral immunity were well documented through the Toll and IMD pathways (De Gregorio et al. 2002). According to An et al. (2013) melanization regulated the innate immunity via binding, eradicated the interfering organisms, and intervened using a SP cascade that is regulated by serpins in a similar manner. Zhao et al. (2012) demonstrated that serpins may be associated with the management of the innate immunity of different

insects on the basis of genome wide identification. The process of melanization combined with other immune activity, such as blood coagulation, phagocytosis, wound healing, and antimicrobial peptide expression (Kanost and Gorman 2008).

Drosophila melanogaster has been extensively used to study molecular mechanisms that are involved in the activation and regulation of innate immunity. However, scanty knowledge was found compared with the silkworm, *Bombyx mori*, the tobacco hornworm, *Manduca sexta*, the and *Tenebrio molitor* for the performance of the Toll signaling pathway (An et al. 2009; Lindsay and Wasserman 2014). In the melanization process, phenoloxidase (PO) catalyzes the hydroxylation of monophenols to o-diphenols and the oxidation of o-diphenols to quinones, which polymerize to form melanin (Nappi et al. 2009) and inactive zymogens called prophenoloxidase (PPO) (Cerenius et al. 2008). Other researchers reported that PPO activation is mediated by a SP cascade (serpin superfamily), which contains 400 amino acid residues with an exposed reactive center loop near their carboxyl terminus and functions as suicide substrate inhibitors by forming irreversible complexes with target proteases after the cleavage of a scissile bond (designated P1–P1′) in the reactive center loop (Gettins 2002; Jiang et al. 2009; An et al. 2013). Recently, An et al. (2013) reported the functional role of SP inhibitors (SPn27A, MP2) in *Drosophila* and stated that both are used in the prevention of prophenoloxidase-1. Thus, they concluded that molecular and biochemical analyses play a vital role in understanding the PPO-activating cascade in insects and expect to shed some light on the action of protease, which could be beneficial in targeting biochemical pathways that are potentially applicable to control of the viral infection.

8.8 Molecular and Cell Target-Based Analysis of Antiviral Protein (Silkworm, *Drosophila*, Anopheles)

8.8.1 Genome-Wide Identification and Expression

For the study of genome-wide identification and expression analysis, there is a current need to understand the genetics and regulatory mechanisms of immune responses of insects that have developed the effective biological control system (You et al. 2013). Recently, Xia et al. (2015) investigated the genome-wide identification, characterization, and expression analysis in *Plutella xylostella* by using Toll, IMD, and JAK-STAT signaling pathways. Previously, genome-wide identification and expression were reported by Zhao et al. (2010) in *Bombyx mori* who compared the expression analysis of SPs and their homologs (Table 8.1). SP homologs (SPHs) are similar to SPs in amino acid sequences, but they have no protease activity because of the loss of one or more of the catalytic residues (Zou et al. 2006). In insects, SPHs participate in the innate immune response to regulate the mechanism insect immunity (Dimopoulos et al. 1997; Kim et al. 2008). Genome-wide characterization was also performed in SP and SP homologs of *Drosophila melanogaster* (Ross et al. 2003). Besides these insects, *Anopheles gambiae* and

Table 8.2 List of serine proteases and serine protease inhibitors which upregulated and downregulated the transcripts region and their gene expression analysis (adopted from Riddell et al. 2014)

	Upregulated	Downregulated
Serine proteases		
cSp3	BTT35293_1	BTT10579_1, BTT10912_1, BTT25711_1
Sp18	BTT20808_1	BTT20808_1
Sp27	BTT40251_1	BTT40251_1
Sp28		BTT20637_1
Sp35	BTT05300_1	BTT10155_1
Sp40	BTT15256_1	
Sp23	BTT01709_1, BTT05886_1, BTT09081_1, BTT20661_1, BTT20725_1, BTT24359_1, BTT25071_1	
Serine protease homologs		
cSPH39		BTT21868_1
Sph54		BTT27769_1
Sph56	BTT17814_1	
Serine protease inhibitors		
Kunitz ser-protease inhibitor		BTT14993_1
Necrotic (nec)	BTT35742_1	
Spn 4	BTT04130_1	BTT04130_1
SRPN10		BTT02607_1, BTT4508_1, BTT20259_1

Apis mellifera also had immunity-related SPs and SPH characteristics (Christophides et al. 2002; Zou et al. 2006). There is little information about these proteins in *Bombyx mori* against SPs and SPHs, but it emphasizes the biological functions such as digestion, immune response, development (Nakazawa et al. 2004). Furthermore, Tanaka et al. (2008) identified the potential immunity of SPs and SPH-related geneswhile modifications to the mRNA level that were considered to be involved in the antiviral mechanism. According to (Zhao et al. 2010; Xia et al. 2007; and Riddell et al. 2014) the SPs and SPH genes were quietly used in upregulation and downregulation after pathogen induction by using microarray and real-time quantitative experiments (Table 8.2).

8.8.2 cDNA Microarray-Based Assay and Two-Dimensional Gel Electrophoresis

In recent years, Chang et al. (2011) studied the comparative analysis of gene expression techniques and determined that cDNA microarray and two-dimensional gel electrophoresis have become part of routine in checking the changes in gene

expression. Among them, such methodologies are implemented to identify differently expressed transcripts by virtue of many genes being examined simultaneously. Recently, several studies have been conducted on insect resistance such as SP-2, lipase-1, and alkaline trypsin protein purified from the digestive juice of *B. mori* larvae showed strong antiviral activity to BmNPV in vitro (Nakazawa et al. 2004; Ponnuvel et al. 2003, 2012).

In an earlier report, the SP gene in *Bombus ignites* was cloned to provide some valuable knowledge on determining the possible function/role(s) of SPs (Choo et al. 2010). In this report, Choo et al. first isolated the genomic DNA and PCR of the *BiSP* gene and southern blot analysis, then expression of recombinant BiSP protein, preparation of polyclonal antibody, and western blot analysis; further, cDNA encoding BiSP was expressed as a 28-kDa polypeptide in baculovirus-infected insect cells, and the recombinant BiSP showed activity in a protease enzyme assay. BiSP was specifically expressed in the midgut of *B. ignitus* queens, males, and workers, suggesting that the BiSP is a gut enzyme involved in the digestion of dietary proteins, i.e., SP of *B. ignitus*, but the involvement of BiSP in the defense from microbial infection. Choi et al. (2006) constructed the cDNA library by screening, sequencing, and generating the expressed sequence tags (ESTs). Altschul et al. (1997) also compared the sequence by using DNASIS and BLAST software and used this to align the amino acid sequences of SP. In accordance with this hypothesis, we also interpreted our data by using a sequence comparison program and identified the pathogen that was responsible for the infection. Using the fluorescent differential display (FDD) technique, Bmsop2 and Bms3a were identified (Xu et al. 2012).

8.8.3 Polyclonal Antibody-Based Preparation and Western Blot Analysis

Polyclonal antibody-based analysis is used to identify immune mechanisms in insects, as SPs act as a pro-PO cascade, which is involved in superoxide generation, melanin synthesis, and subsequent sequestration of foreign matter entering the hemocoel of the insect (Ashida and Brey 1998). Another SP cascade in insects is involved in the establishment of the dorso-ventral pattern in the *Drosophila* embryo (Morisato 1995). Choo et al. (2007) reported that the *B. ignitus* SP (BiSP) gene functioned as a gut enzyme involved in the digestion of dietary proteins by polyclonal antibody-based preparation and western blotting. The recombinant proteins fused with an N-term GST tag were overexpressed in *E. coli* and further purified to near homogeneity to prepare mouse antibodies. The western blot analysis showed that these proteins were expressed in various tissues and organs, and in different developmental stages. Amazingly, the expression of BmTHY2 was hugely increased during the pupae stage, indicating a specialized role during this period. The expression of these proteins was gradually decreased in BmN cells infected by BmNPV, suggesting that they might play different roles in virus infection (Ma et al. 2015).

8.9 (In) Multigene Expression

In the present scenario, multiple gene expression techniques used on the basis of three potential criteria:

1. The functions of targeted genes exclusively related to multiple genes.
2. Break down of the co-expression of multiple genes or multi-functional protein complex in silk gland bioreactor research.
3. Construction of multiresistant transgenic *B. mori* strains such as BmNPV and cytoplasmic polyhedrosis virus (BmCPV).
4. For the production of silk material genetically modified techniques are used, which improve the middle and posterior silk gland in *B. mori*. (Wang et al. 2017). Previously, an applied aspect of silkworm on genome sequencing projects resulted in a higher requirement of functional genomic research, which is providing a suitable platform for the study of multiple genes (Xia et al. 2009). Recently, Wang et al. (2017) reported 2A self-cleaving peptide-based multiple gene expression system in the *B. mori* and concluded that the multiple gene expression would be an efficient tool of the functional genomic era. In the process, he investigated that the gene regulated the simultaneous expression and cleavage of multiple gene targets in the silk gland of transgenic silkworms. First, a glycine-serine-glycine spacer (GSG) was found to significantly improve the cleavage efficiency of 2A. This study enhances the functional knowledge of genes and proteins, and potentially advances innovative research into various functional silk materials in medicine, cosmetics, and other biomedical areas.

8.10 Differentially Expressed Genes Analysis

In recent years, there has been a need to analyze gene efficiency by comparing the transcriptome of two strains, which is used to improve the efficiency and gene discovery of host–pathogen responses. A new technique was developed by scientists and used for comparative analysis of two strains with a transcriptome (Wang et al. 2016). According to their hypothesis, they reported that molecular changes in *B. mori* occurred during BmNPV infection and were examined by transcriptome sequencing in the isogenic line BC9 and the recurrent parent P50. These genes were related to transport, virus replication, intracellular innate immune, and apoptosis (Wang et al. 2016). After normalizing gene expression levels, DEGs were obtained by pair-wise comparison of the four transcriptome libraries using IDEG6 software (Romualdi et al. 2003). Genes participating in innate immunity pathways were identified and analyzed with regard to their potential role in BmNPV infection in silkworm, which could be classified into the Toll pathway, the IMD pathway, the PPO pathway, the pattern recognition receptor, and the antimicrobial peptide. Wang et al. (2016) further reported that the SP inhibitor performed a significant role in the PPO pathway and it appears that there was downregulation in the gene of 47% and 57% respectively, whereas 30% and 20% were upregulated after infection with

BmNPV, and 57% were downregulated and 20% were upregulated in P50 after BmNPV infection. By this comparative analysis, a total of 869 DEGs were obtained, which included many genes potentially related to BmNPV resistance. After that, Wang et al. (2016) predicted that reliable evidence may be produced to clarify the molecular mechanism of the silkworm. Hu et al. (2014) also reported analysis of several differentially expressed genes (DEGS), which is involved in metabolism, immunity, and inflammatory responses in *Microtus fortis* following infection with *Schistosoma japonicum* based on comparative transcriptome analysis.

Conclusion

In the current scenario, wonderful progress has been made in the antiviral mechanism of SP against viral infection. The synergism of host and pathogen played a pivotal role in viral infections, and various mechanisms such as genome-wide identification and expression, microarray-based expression, recombinant-based expression, polyclonal antibody-based expression, multiple gene-based expression, etc., were performed using the SP pathway. The discovery of Molecular and cellular analysis - Genome-Wide Identification and Expression, cDNA Microarray-based Assay, Polyclonal Antibody-Based Preparation and Western Blot, Multigene Expression, Differentially expressed genes analysis of anti-protein detection plays a vital role in fighting viral infection, but recently, antimicrobial target-based pathways, immensely used, such as siRNA, Toll, JAK-STAT, IMD, etc., is used to determine the host–pathogen-specific immune response to the fascinating antiviral mechanism. In this study, a new technology was developed for understanding the fundamental and applied aspects of insect immunity against viral infection in the functional genomic era, and potentially improving the antiviral mechanism.

References

Alto BW, Bettinardi D (2013) Temperature and dengue virus infection in mosquitoes: independent effects on the immature and adult stages. Am J Trop Med Hyg 88:497–505

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSIBLAST: a new generation of protein database search programs. Nucl Acids Res 25:3389–3402

An C, Ishibashi J, Ragan EJ, Jiang H, Kanost MR (2009) Functions of Manduca sexta hemolymph proteinases HP6 and HP8 in two innate immune pathways. J Biol Chem 284:19716–19726

An C, Zhang M, Chu Y, Zhangwu Zhao Z (2013) Serine protease MP2 activates prophenoloxidase in the melanization immune response of drosophila. PLoS One 8(11):1–10

Ashida M, Brey PT (1997) Recent advances in research on the insect prophenoloxidase cascade. In: Brey PT, Hultmark D (eds) Molecular mechanisms of immune responses in insects. Chapman and Hall, London, pp 135–172

Ashida M, Brey PT (1998) In: Brey PT, Hultmark D (eds) Molecular mechanisms of immune responses in insects. Chapman & Hall, London, pp 135–172

Bao YY, Tang XD, Lv ZY, Wang XY, Tian CH, YP X, Zhang CX (2009) Gene expression profiling of resistant and susceptible Bombyx mori strains reveals nucleopolyhedrovirus-associated variations in host gene transcript levels. Genomics 94(2):138–145

Barett AJ (1994) Proteolytic enzymes: serine and cysteine peptidases. Methods Enzymol 244:1–15
Beier JC (1998) Malaria parasite development in mosquitoes. Annu Rev Entomol 43:519–543
Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL et al (2013) The global distribution and burden of dengue. Nature 496:504–507
Cerenius L, Lee BL, Soderhall K (2008) The proPO-system: pros and cons for its role in invertebrate immunity. Trends Immunol 29:263
Chang C, Liu X, Chen K (2011) Molecular cloning, expression and characterization of a novel gene**β**-N-acetylglucosaminidase from Bombyx mori. Adv Biosci Biotechnol 2(2):123–127
Chen J, X-F W, Zhang YZ (2006) Expression, purification and characterization of human GM-CSF using silkworm pupae (Bombyx morie) as a bioreactor. J Biotechnol 123:236–247
Cheng Y, Wang X-Y, Hao H, Killiny N, Jia-Ping X (2014) A hypothetical model of crossing Bombyx mori nucleopolyhedrovirus through Its host midgut physical barrier. PLoS One 9(12):e115032
Choi YS, Lee KS, Yoon HJ, Kim I, Sohn HD, Jin BR (2006) A bumblebee thioredoxin-like protein gene that is upregulated by a temperature stimulus and lipopolysaccharide injection. Eur J Entomol 103:291–296
Choo YM, Lee KS, Yoon HJ, Lee SB, Kim JH, Sohn HD, Jin BR (2007) A serine protease from the midgut of the bumblebee, Bombus ignitus (hymenoptera: apidae): cDNA cloning, gene structure, expression and enzyme activity. Eur J Entomol 104:1–7
Choo YM, Lee KS, Yoon HJ, Kim BY, Sohn MR, Roh JY, Je YH, Kim NJ, Kim I, Woo SD, Sohn HD, Jin BR (2010) Dual function of a bee venom serine protease: prophenoloxidase-activating factor in arthropods and fibrin(ogen)olytic enzyme in mammals. PLoS One 5(5):1–10
Christophides GK, Zdobnov E, Barillas-Mury C, Birney E, Blandin S, Blass C, Brey PT, Collins FH, Danielli A, Dimopoulos G (2002) Immunity-related genes and gene families in Anopheles gambiae. Science 298(5591):159–165
Danielli A, Loukeris TG, Lagueux M, Muller HM, Richman A, Kafatos FC (2000) A modular chitin-binding protease associated with hemocytes and hemolymph in the mosquito Anopheles gambiae. Proc Natl Acad Sci U S A 97:7136–7141
De Gregorio E, Han SJ, Lee WJ, Baek MJ, Osaki T et al (2002) An immune-responsive Serpin regulates the melanization cascade in Drosophila. Dev Cell 3:581
Dimopoulos G, Richman A, Mullar HM, Kafatos FC (1997) Molecular immune responses of the mosquito Anopheles gambiae to bacteria and malaria parasites. Proc Natl Acad Sci U S A 94:11508–11513
Engelhard EK, Volkman LE (1995) Developmental resistance in fourth instar Trichoplusia ni orally inoculated with Autographa californica M nuclear polyhedrosis virus. Virology 209:384–389
Feng Fan, Hu Ping and Chen Keping (2013) Progress of antiviral mechanisms in the mulberry silkworm: A review 7(14):1173–1178
Felfoldi G, Eleftherianos I, Ffrench-Constant RH, Venekei I (2011) A serine proteinase homolog, SPH-3, plays a central role in insect immunity. J Immunol 186:4828–4834
Fragkoudis R, Chi Y, Siu RW, Barry G, Attarzadeh-Yazdi G, Merits A, Nash AA, Fazakerley JK, Kohl A (2008) Semliki forest virus strongly reduces mosquito host defense signaling. Insect Mol Biol 17:647–656
Fragkoudis R, Attarzadeh-Yazdi G, Nash AA, Fazakerley JK, Alain Kohl A (2009) Advances in dissecting mosquito innate immune responses to arbovirus infection. J Gen Virol 90:2061–2072
Gabay JE (1994) Antimicrobial proteins with homology to serine protease. Ciba Found Symp 186:237–247
Gabay JE, Almeid RP (1993) Antibiotic peptides and serine protease homologs in human polymorphonuclear leukocytes: defensins and azurocidin. Curr Opin Immunol 5:97–102
Gettins PG (2002) Serpin structure, mechanism, and function. Chem Rev 102:4751
Gorman MJ, Paskewitz SM (2001) Serine proteases as mediators of mosquito immune responses. Insect Mol Biol 31:257–262
Gorman MJ, Andreeva OV, Paskewitz SM (2000a) Sp22D: a multidomain serine protease with a putative role in insect immunity. Gene 251:9–17

Gorman MJ, Olga V, Andreeva OV, Susan M, Paskewitz SM (2000b) Molecular characterization of five serine protease genes cloned from Anopheles gambiae hemolymph. Insect Biochem Mol Biol 30:35–46

Hartley BS (1960) Proteolytic enzymes. Annu Rev Biochem 29:45–72

He WY, Zheng YP, Tang L, Zheng SC, Beliveau C, Doucet D, Cusson M, Feng QL (2009) Cloning, expression and localization of a trypsin-like serine protease in the spruce budworm, Choristoneura fumiferana. Insect Sci 16:455–464

Hedstrom L (2002) Serine protease mechanism and specificity. Chem Rev 102:4501–4524

Herrero S, Combes E, Oers V, Vlak MM, DeMaagd RA, Beekwilder J (2005) Identification and recombinant expression of a novel chymotrypsin from Spodoptera exigua. Insect Biochem Mol Biol 35:1073–1082

Holt RA, Subramanian GM, Halpern A, Sutton GG, Charlab R, Nusskern DR, Wincker P, Clark AG, Ribeiro JM, authors o (2002) The genome sequence of the malaria mosquito Anopheles gambiae. Science 298:129–149

Hu Y, YX X, WY L, Yuan ZY, Quan H, Shen YJ et al (2014) De novo assembly and transcriptome characterization: novel insights into the natural resistance mechanisms of Microtus fortis against Schistosoma japonicum. BMC Genomics 15(1):1–13

Huang HS, Wang H, Lee SY, Johansson WM, Soderhall K, Cerenius L (2000) A cell adhesion protein from the crayfish Pacifastacus leniusculus, a serine protease homologue similar to drosophila masquerade. J Biol Chem 275:9996–10001

Iwanaga S (2002) The molecular basis of innate immunity in the horseshoe crab. Curr Opin Immunol 14:87–95

Jiang R, Kim EH, Gong JH, Kwon HM, Kim CH et al (2009) Three pairs of protease-serpin complexes cooperatively regulate the insect innate immune responses. J Biol Chem 284:35652

Jupatanakul N, Shuzhen Sim S, Angleroa-Rodroaguez YI, Souza-Neto J, Das S, Poti KE, Rossi SL, Bergren N, Vasilakis N, Dimopoulos G (2017) Engineered *Aedes aegypti* JAK/STAT pathway-mediated immunity to dengue virus. PLoS Negl Trop Dis 1:24

Kaji K, Tomino S, Asano T (2009) A serine protease in the midgut of the silkworm, Bombyx mori: protein sequencing, identification of cDNA, demonstration of its synthesis as zymogen form and activation during midgut remodeling. Insect Biochem Mol Biol 39:207–217

Kanost MR, Gorman MJ (2008) Phenoloxidases in insect immunity. In: Beckage NE (ed) Insect immunology. Elsevier/Academic, Manhattan, pp 69–96

Katsuma S, Daimon T, Horie S, Kobayashi M, Shimada T (2006) N-linked glycans of Bombyx mori nucleopolyhedrovirus fibroblast growth factor are crucial for its secretion. Biochem Biophys Res Commun 350(4):1069–1075

Kim CH, Kim SJ, Kan H, Kwon HM, Roh KB et al (2008) A three-step proteolytic cascade mediates the activation of the peptidoglycan-induced toll pathway in an insect. J Biol Chem 283:7599–7607

Kim SY, Jeong EJ, Song J, Park KS (2009) Molecular cloning and characterization of a serine protease-like protein from silkworm (Bombyx Morie). Gene Genom 31(5):387–395

Kotani E, Niwa T, Tokizane M, Suga K, Sugimura Y, Oda K, Mori M, Furusawa T, (1999) Cloning and sequence of a cDNA for a highly basic protease from the digestive juice of the silkworm, *Bombyx mori.* Insect Mol. Biol. 8:299–304

Kwon TH, Kim MS, Choi HW, Joo CH, Cho MY, Lee BL (2000) A masquerade-like serine protease homologue necessary for phenoloxidase activity in the coleopteran insect Holotrichia diomphalia larvae. Eur J Biochem 267:6188–6196

Lavine MD, Strand MR (2002). Insect hemocytes and their role in immunity. Insect Biochem. Mol. Biol. 32(10):1295–1309

Lehane MJ, Aksoy S, Levashina E (2004) Immune responses and parasite transmission in blood-feeding insects. Trends Parasitol 20(9):433–439

Lemaitre B, Hoffmann J (2007) The host defense of Drosophila melanogaster. Annu Rev Immunol 25:697–743

Lin H, Lin X, Zhu J, XO Y, Xia X, Yao F, Guang Yang G, You M (2017) Characterization and expression profiling of serine protease inhibitors in the diamondback moth, Plutella xylostella (Lepidoptera: Plutellidae). BMC Genomics 18:162

Lindsay S, Wasserman SA (2014) Conventional and non-conventional Drosophila Toll signaling. Dev Comp Immunol 42(1):10.10–10.16

Liu HW, Li YS, Tang X, Guo PC, Wang DD, Chun-Yan Zhou CY, Xia QY, Zhao P (2016) A midgut-specific serine protease, BmSP36, is involved in dietary protein digestion in the silkworm, Bombyx mori. Insect Sci:1–15. https://doi.org/10.1111/1744-7917.12369

Liu Y, Sui YP, Wang JX, Zhao XF (2009) Characterization of the trypsin-like protease (Ha-TLP2) constitutively expressed in the integument of the cotton bollworm, Helicoverpa armigera. Arch Insect Biochem Physiol 72:74–87

Ma S, Kang Z, Lü P, Yang Y, Yao Q, Xia H, Chen K (2015) Molecular and physiological characterization of two novel multirepeat **β**-thymosins from silkworm, Bombyx mori. PLoS One 10(10):e0140182

Miao XX, SJ X, Li MH, Li MW, Huang JH, Dai FY, Marino SW, Mills DR, Zeng PY, Mita K, Jia H, Zhang Y, Liu WB, Xiang H, Guo QH, AY X, Kong XY, Lin HX, Shi YZ, Lu G, Zhang XL, Huang W, Yasukochi Y, Sugasaki T, Shimada T, Nagaraju J, Xiang ZH, Wang SY, Goldsmith MR, Lu C, Zhao GP, Huang YP (2005) Simple sequence repeat-based consensus linkage map of Bombyx mori. Proc Natl Acad Sci U S A 102:16303–16308

Monsma SA, Oomens AG, Blissard GW (1996) The GP64 envelope fusion protein is an essential baculovirus protein required for cell-to-cell transmission of infection. J Virol 70(7):4607–4616

Morisato D (1995) Signaling pathways that establish the dorsal-ventral pattern of the Drosophila embryo. Annu Rev Genet 29:371–399

Murugasu-Oei B, Rodrigues V, Yang X, Chia W (1995) Masquerade: a novel secreted serine protease-like molecule is required for somatic muscle attachment in the drosophila embryo. Genes Dev 9:139–154

Nakamura T, Nishizawa T, Hagiya M, Seki T, Shionishi M, Sugiura A, Tashiro K, Shiizu S (1989) Molecular cloning and expression of human hepatocyte growth factor. Nature 342:440–443

Nakazawa H, Tsuneishi E, Ponnuvel KM, Furukawa S, Asaoka A, Tanaka H, Ishibashi J, Yamakawa M (2004) Antiviral activity of a serine protease from the digestive juice of Bombyx mori larvae against nucleopolyhedrovirus. Virology 321(1):154–162

Nappi A, Poirie M, Carton Y (2009) The role of melanization and cytotoxic byproducts in the cellular immune responses of drosophila against parasitic wasps. Adv Parasitol 70:99–121

Perona JJ, Craik CS (1995) Structural basis of substrate specificity in the serine proteases. Protein Sci 4(3):337–360

Polgar L (2005) The catalytic triad of serine peptidases CMLS. Cell Mol Life Sci 62:2161–2172

Ponnuvel KM, Nakazawa H, Furukawa S, Asaoka A, Ishibashi J, Tanaka H, Yamakawa M (2003) A lipase isolated from the silkworm Bombyx mori shows antiviral activity against nucleopolyhedrovirus. J Virol 77(19):10725–10729

Ponnuvel KM, Koundinya PR, Sinha RK, Kamble CK (2008) Mechanism of viral resistance in mulberry silkworm, Bombyx mori L. Indian J Seric 47(1):1–6

Ponnuvel KM, Nithya K, Sirigineedi S, Awasthi AK, Yamakawa M (2012) In vitro antiviral activity of an alkaline trypsin from the digestive juice of Bombyx mori larvae against nucleopolyhedrovirus. Arch Insect Biochem 81(2):90–104

Popham HJ, Shelby KS, Brandt SL, Coudron TA (2004) Potent virucidal activity in larval Heliothis virescens plasma against Helicoverpa zea single capsid nucleopolyhedrovirus. J Gen Virol 85(8):2255–2261

Qin L, Xia H, Shi H, Zhou Y, Chen L, Yao Q, Liu X, Feng F, Yuan Y, Chen K (2012) Comparative proteomic analysis reveals that caspase-1 and serine protease may be involved in silkworm resistance to Bombyx mori nuclear polyhedrosis virus. J Proteome 75:3630–3638

Rahman MM, Gopinathan KP (2004) Systemic and in vitro infection process of Bombyx mori nucleopolyhedrovirus. Virus Res 101:109–118

Randall RE, Goodbourn S (2008) Interferons and viruses: interplay between induction, signaling, antiviral responses and virus countermeasures. J Gen Virol 89:1–47

Rao MB, Tanksale AM, Ghatge MS, Deshpande VV (1998) Molecular and biotechnological aspects of microbial protease. Microbial Mol Biol Rev 62:597–615

Rawlings ND, Barrett AJ (2000) MEROPS: the peptidase database. Nucleic Acids Res 28:323–325

Richman AM, Bulet P, Hetru C, Barillas-Mury C, Hoffmann JA et al (1996) Inducible immune factors of the vector mosquito Anopheles gambiae: biochemical purification of a defensin antibacterial peptide and molecular cloning of preprodefensin cDNA. Insect Mol Biol 5:203–210

Riddell CE, Garces JDL, Adams S, Barribeau SM, David Twell D, Eamonn B, Mallon EB (2014) Differential gene expression and alternative splicing in insect immune specificity. BMC Genomics 15(1):1031

Romualdi C, Bortoluzzi S, DAlessi F, Danieli GA (2003) IDEG6: a web tool for detection of differentially expressed genes in multiple tag sampling experiments. Physiol Genomics 12(2):159–162

Ross J, Jiang H, Kanost MR, Wang Y (2003) Serine proteases and their homologs in the Drosophila melanogaster genome: an initial analysis of sequence conservation and phylogenetic relationships. Gene 304:117–131

Saleem M, Rehman A, Yasmin R, Munir B (2012) Biochemical analysis and investigation on the prospective applications of alkaline protease from a Bacillus cereus strain. Mol Biol Rep 39(6):6399–6408

Soares TS, Watanabe RM, Lemos FJ, Tanaka AS (2011) Molecular characterization of genes encoding trypsin-like enzymes from Aedes aegypti larvae and identification of digestive enzymes. Gene 489:70–75

Souza-Neto JA, Sim S, Dimopoulos G (2009) An evolutionary conserved function of the JAK-STAT pathway in anti-dengue defense. Proc Natl Acad Sci U S A 106:17841–17846

Tanaka H, Ishibashi J, Fujita K, Nakajima Y, Sagisaka A, Tomimoto K, Suzuki N, Yoshiyama M, Kaneko Y, Iwasaki T (2008) A genome-wide analysis of genes and gene families involved in innate immunity of Bombyx mori. Insect Biochem Mol Biol 38(12):1087–1110

Verma J, Modi DR, Sharma R, Saxena S (2011) Vital role of alkaline protease in bio-industries: a review. Plant Archives 11(2):1083–1092

Wang H, Blair CD, Olson KE, Clem RJ (2008) Effects of inducing or inhibiting apoptosis on Sindbis virus replication in mosquito cells. J Gen Virol 89:2651–2661

Wang XY, HZ Y, Geng L, JP X, Yu D, Zhang SZ, Ma Y, Dong-Qiong Fei DQ (2016) Comparative transcriptome analysis of Bombyx mori (Lepidoptera) larval midgut response to BmNPV in susceptible and near-isogenic resistant strains. PLoS One 11(5):1–22

Wang Y, Wang F, Riyuan Wang R, Zhao P, Xia Q (2017) 2A self-cleaving peptide-based multi-gene expression system in the silkworm Bombyx mori. Sci Rep 5:16273

Wei Z, Yin YP, Zhang B, Wang ZK, Peng GX, Cao YQ, Xia YX (2007) Cloning of a novel protease required for the molting of Locusta migratoria manilensis. Develop Growth Differ 49:611–621

Xia Q, Zhou Z, Lu C, Cheng D, Dai F, Li B, Zhao P, Zha X, Cheng T, Chai C (2004) A draft sequence for the genome of the domesticated silkworm (Bombyx mori). Science 306(5703):1937–1940

Xia Q, Cheng D, Duan J, Wang G, Cheng T, Zha X, Liu C, Zhao P, Dai F, Zhang Z (2007) Microarray-based gene expression profiles in multiple tissues of the domesticated silkworm, Bombyx mori. Genome Biol 8(8):R162

Xia Q, Guo Y, Zhang Z, Li D, Xuan Z, Li Z (2009) Complete resequencing of 40 genomes reveals domestication events and genes in silkworm (Bombyx morie). Science 326:433–436

Xia X, Liying Yu l, Xue M, Yu X, Vasseur L, Gurr GM, Baxter SW, Lin H, Lin J, You M (2015) Genome-wide characterization and expression profiling of immune genes in the diamondback moth, Plutella xylostella (L.) Sci Rep 5:1–13

Xu QY, Lu AR, Xiao GH, Yang B, Zhang J, Li XQ, Guan JM, Shao QM, Beerntsen BT, Zhang P, Wang CS, Ling EJ (2012) Transcriptional profiling of midgut immunity response and degeneration in the wandering silkworm, Bombyx mori. PLoS One 7:43769

You M et al (2013) A heterozygous moth genome provides insights into herbivory and detoxification. Nat Genet 45:220–225

Zdobnov EM, Von Mering C, Letunic I, Torrents D, Suyama M, Copley RR, Christophides GK, Thomasova D, Holt RA, Subramanian GM, Mueller HM, Dimopoulos G, Law JH, Wells MA, Birney E, Charlab R, Halpern AL, Kokoza E, Kraft CL, Lai Z, Lewis S, Louis C, Barillas-Mury C, Nusskern D, Rubin GM, Salzberg SL, Sutton GG, Topalis P, Wides R, Wincker P, Yandell M, Collins FH, Ribeiro J, Gelbart WM, Kafatos FC, Bork P (2002) Comparative genome and proteome analysis of Anopheles gambiae and Drosophila melanogaster. Science 298:149–159

Zhao P, Wang GH, Dong ZM, Duan J, PZ X, Cheng TC, Xiang ZH, Xia QY (2010) Genome-wide identification and expression analysis of serine proteases and homologs in the silkworm Bombyx mori. BMC Genomics 11:405

Zhao P, Dong ZM, Duan J, Wang GH, Wang LY, Li YS, Xiang ZH, Xia QY (2012) Genome-wide identification and immune response analysis of serine protease inhibitor genes in the silkworm, Bombyx mori. PLoS One 7(2):e31168

Zou Z, Lopez DL, Kanost MR, Evans JD, Jiang H (2006) Comparative analysis of serine protease-related genes in the honey bee genome: possible involvement in embryonic development and innate immunity. Insect Mol Biol 15(5):603–614

9 Preventive, Diagnostic and Therapeutic Applications of Baculovirus Expression Vector System

Neeraj Kumar, Deepak Pandey, and Ashutosh Halder

Abstract

Different strategies are being worked out for engineering the original baculovirus expression vector (BEV) system to produce cost-effective clinical biologics at commercial scale. To date, thousands of highly variable molecules in the form of heterologous proteins, virus-like particles, surface display proteins/antigen carriers, heterologous viral vectors and gene delivery vehicles have been produced using this system. These products are being used in vaccine production, tissue engineering, stem cell transduction, viral vector production, gene therapy, cancer treatment and development of biosensors. Recombinant proteins that are expressed and post-translationally modified using this system are also suitable for functional, crystallographic studies, microarray and drug discovery-based applications. Till now, four BEV-based commercial products (Cervarix®, Provenge®, Glybera® and Flublok®) have been approved for humans, and myriad of others are in different stages of preclinical or clinical trials. Five products (Porcilis® Pesti, BAYOVAC CSF E2®, Circumvent® PCV, Ingelvac CircoFLEX® and Porcilis® PCV) got approval for veterinary use, and many more are in the pipeline. In the present chapter, we have emphasized on both approved and other baculovirus-based products produced in insect cells or larvae that are important from clinical perspective and are being developed as preventive, diagnostic or therapeutic agents. Further, the potential of recombinant adeno-associated virus (rAAV) as gene delivery vector has been described. This system, due to its relatively extended gene expression, lack of pathogenicity and the ability to transduce

N. Kumar (✉) • D. Pandey • A. Halder
Department of Reproductive Biology, All India Institute of Medical Sciences, New Delhi 110029, India
e-mail: drnknirwal@aiims.ac.in

D. Kumar, C. Gong (eds.), *Trends in Insect Molecular Biology and Biotechnology*, https://doi.org/10.1007/978-3-319-61343-7_9

a wide variety of cells, gained extensive popularity just after the approval of first AAV-based gene therapy drug alipogene tiparvovec (Glybera®). Numerous products based on AAV which are presently in different clinical trials have also been highlighted.

9.1 Introduction

Baculovirus (family: *Baculoviridae*) derived its name from the Latin word "baculum" meaning "stick". They are rod-shaped (30–60 × 250–300 nm) large enveloped viruses with circular, supercoiled double-stranded DNA genomes, approximately 80–180 kb in size. While most of the baculoviruses infect their natural host, i.e., butterflies and moths (*Lepidoptera*), few are also known to infect sawflies (*Hymenoptera*) and mosquitoes (*Diptera*) (King et al. 2011). They have not been linked with any disease in any organism outside the phylum *Arthropoda* (Kost and Condreay 2002). Baculoviruses are well known for their role as biopesticides and are efficient tools for heterogeneous protein production in insect cells (Summers 2006). Morphologically, these enveloped viruses have been classified into two phenotypes: occlusion-derived viruses (ODVs) that are embedded in paracrystalline matrix forming polyhedral occlusion bodies (OBs) which are responsible for horizontal transmission between insects and the budded viruses (BuVs) present in the haemolymph which spreads infection from cell to cell (Luckow and Summers 1988). Occlusion body morphology was initially used to define two major groups of baculoviruses: nucleopolyhedroviruses (NPVs) and the granuloviruses (GVs). NPVs obtain their envelop from host nuclear membrane and are occluded within main occlusion protein polyhedrin forming large (1–15 μm) polyhedral inclusion bodies, while GVs obtain their envelop from cell membrane to make oval-shaped single virion structure called granule or capsule with diameter in the range of 0.2–0.4 μm (King et al. 2011). NPVs are further distinguished as single nucleopolyhedrovirus or multiple nucleopolyhedrovirus based on the number of nucleocapsids in a polyhedral inclusion body (O'Reilly et al. 1994). OBs allow virions to remain infectious for long period due to their highly resistant and stable structure.

Baculovirus-infected insect cell expression system has been used for the routine production of recombinant proteins, including several proteins of therapeutic nature over the last three decades. The establishment of this system begins from the production of human beta interferon (INF-β), the protein normally not produced in the cultured human cells. It was produced with a recombinant *Autographa californica* multiple nucleopolyhedrovirus (AcMNV) by exploiting its polyhedrin promoter (Smith et al. 1983). In this system, the protein coding sequence of human interferon gene was linked to the AcNPV polyhedrin gene promoter. The interferon gene was inserted at different positions relative to the AcNPV polyhedrin transcriptional and translational signals. The interferon-polyhedrin hybrid plasmid was then transferred to infectious AcNPV expression vectors by recombination within *S. frugiperda* insect cells, where more than 95% of biologically active glycosylated interferon was produced in the secreted form.

At the same time, another group successfully expressed *Escherichia coli* β-galactosidase gene in insect cells by using this system. A 9.2 kb plasmid construct was made of β-galactosidase gene (1 kb) after fusion with the N-terminal region of the polyhedrin gene (1.2 kb) of AcNPV. Co-transfection of this fused plasmid construct with wild-type AcNPV genomic DNA (134 kb) was performed in order to insert the foreign gene into the polyhedrin gene of AcNPV genome by the process of homologous recombination. Finally, the recombinant viruses were selected as blue plaques in the presence of β-galactosidase indicator X-gal medium. These discoveries mark the beginning of baculovirus expression system, facilitating the engineering and improvement of baculovirus vectors, modification of the sugar moieties of glycoproteins expressed in insect cells and scale up of the cell culture processes.

A baculovirus expression vector (BEV) platform has been tailored by taking advantage of baculoviruses' natural tendency to infect insect cells. There are almost 500 different types of baculoviruses, all of which specifically infect invertebrates. For laboratory research and manufacturing purposes, the most commonly studied baculovirus is *Autographa californica* multiple nucleopolyhedrovirus (*Ac*MNPV) which is often considered as a prototype of baculoviruses. It has a double-stranded circular DNA genome of 134 kb inside a rod-shaped nucleocapsid of size 25 × 260 nm (Fauquet et al. 2005). Its large size genome gives sufficient ability to accommodate a large foreign DNA or multiple genes together.

Typically, recombinant BEVs are constructed by co-transfecting a mixture of transfer plasmid and modified non-infectious and linearized *Ac*MNPV that lacks parental polyhedrin gene and a portion of ORF1629. Transfer plasmid contains the gene of interest (GOI), flanked upstream by strong polyhedrin or p10 promoter and downstream by an essential portion of ORF1629 of *Ac*MNPV for high-level protein expression in insect cells. The transfer plasmid and modified linearized *Ac*MNPV DNA undergo homologous recombination to generate de novo recombinant baculoviruses. After plating of these baculoviruses, single pure plaques of recombinant baculovirus are selected. Subsequently, this plaque is passaged through multiple rounds of insect cell infection to generate a high-titre stock. It creates a working virus bank (WVB) for utilization during downstream processes.

This system was further enhanced for manufacturing and commercialization purposes by multiple ways and technologies. Bacmid technology (Bac to Bac®, Life Technologies) was employed for the generation of recombinant *Ac*MNPV genomes in bacterial host system *E. coli*. FlashbackTM (Oxford Expression Technologies Ltd.) and BacMagicTM (Merck) BaculoOneTM (PAA) technologies are used to avoid the bacterial sequences in the final vector or rapid production of multiple recombinant viruses in a one-step procedure. MultiBac system is being used for the synthesis of multisubunit protein complex and OmniBac as multigene transfer vector for universal generation of recombinant baculoviruses. Sleeping beauty or PiggyBac transposon system are being exploited in highly efficient seamless excision of transposons from the genomic DNA and for its potential to target integration events to desired DNA sequences. For the production of AcMNPV vectors and

recombinant proteins, the *Spodoptera frugiperda Sf21* and its subclone *Sf9* and High Five cell lines are being used. These insect cells exhibit several properties like rapid growth, stress resistance and robust expression of recombinant proteins that make them suitable for the production of clinical biologics and commercial products.

Initially, insect-derived baculovirus expression vector (BEV) was recognized as a safe system for routine production of recombinant proteins both in insect and mammalian cells. During the last three decades, it has emerged as an effective tool for research as well as various applications in the field of biotechnology. It has shown tremendous potential as preventive, diagnostic and therapeutic agent against a myriad of diseases in the form of vaccination, tissue engineering, stem cell transduction, viral vector production and gene therapy (Airenne et al. 2013). It has been extensively used for functional studies, crystallography, biosensors, protein microarray and drug discovery. All these applications are based on different baculovirus-derived products such as heterologous proteins, protein/antigen displayed on baculovirus particle surface, heterologous viral vectors and gene delivery vehicles for mammalian cells (van Oers et al. 2015). In this chapter, we have presented the application of these products from a clinical point of view in three main categories, viz. preventive, diagnostic and therapeutic agents. Most of the approved biomolecules produced by using baculovirus expression system in insect cells have been discussed. As thousands of other products are being developed by BEVs, it seems ineffectual to include the entire list under the ambit of the present chapter; however few among them have been mentioned to have an understanding about the scope of this powerful expression system in the near future.

9.1.1 BEVs As Disease Preventive Agents

BEV exhibits many characteristics that make it suitable for the production of heterologous proteins in insect cells. It can be easily handled in the BSL1/2 laboratories due to its harmless nature to nontarget organisms. These viruses are environmentally safe due to their instability outside the laboratory. It is used to produce high level of proteins in insect cells or larvae where the eukaryotic environment provides the appropriate post-translational modifications. BEVs host insect cells are mostly free of human pathogens and do not require controlled oxygenic environment for their growth. Insect cells can be grown into serum-free medium, and the heterologous protein production can be enhanced to the level of pilot plant or larger bioreactors. Therefore, the proteins obtained by the BEVs can be used as vaccines either in the form of heterologous subunit proteins or virus-like particles (VLPs) formed by subunit proteins of virus.

Subunit vaccines are relatively safe as they are devoid of virus genetic material but exhibit poor immunogenicity that might be due to incorrect folding of the target protein. Structural proteins of viruses such as capsid and envelop proteins assemble into particulate structure similar to the naturally occurring virus or subviral particles. Therefore, virus-like particles (VLPs) that are non-infectious and

non-replicating due to the absence of viral genetic material can be produced in heterologous system (Yamaji 2014). VLPs are highly effective in eliciting both humoral and cellular immune response because of their densely repeated display of viral antigens in right conformation (Roy and Noad 2008). VLPs comparatively exhibit wide spectrum of clinical applications such as prevention of disease as vaccines, diagnostics as antigens for the detection of antibodies and therapeutics in the form of therapeutic vaccines and delivery agents. The use of heterologous proteins and VLPs as preventive agents in the form of vaccines against different diseases is being described (Table 9.1).

A decade back, only two veterinary products were manufactured using BEVs to prevent classical swine fever in pigs. Now, five more new vaccines have been approved, two of which are for humans, and many more products are in the development phase. Here, approved vaccines as well as development of other vaccines in preclinical stages have been highlighted.

9.1.1.1 Veterinary Vaccines

(a) *Subunit marker vaccine for classical swine fever*: Classical swine fever virus (CSFV) infection invariably develops antibodies against virus envelop proteins E^{RNS} and glycoprotein E2 and the non-structural protein NS3 in swine (Paton et al. 1991). However, injection of only glycoprotein E2 in pigs is reported to sufficiently provide protection to CSFV (Van Rijn et al. 1996). Therefore, a subunit vaccine has been produced on the basis of conserved glycoprotein E2 with a baculovirus vector in insect cells (Moormann et al. 2000). Glycoprotein E2 being expressed as envelop protein, its C-terminal transmembrane domain was removed to secrete it into the medium, and the residual baculovirus was inactivated with 2-bromoethyl-imminebromide. This vaccine was manufactured and commercialized as "Porcilis Pesti®" by MSD Animal Health. The same vaccine was also commercialized as "BAYOVAC CSF E2®/Advasurea" by Bayer AG/Pfizer Animal Health but was later discontinued.

(b) *Virus-like particle (VLP)-based vaccine for porcine circovirus type 2*: Porcine circovirus type 2 (PCV2) vaccine was developed based on VLPs. PCV2 is the primary causative agent of postweaning multisystemic wasting syndrome (PMWS) in swine. Two major open reading frames (ORF1 and ORF2) have been identified in PCV2. ORF2 encodes a major structural protein with type-specific epitopes and is found to be highly immunogenic. Therefore, ORF2 that encodes the capsid protein was used to develop the vaccine with a baculovirus in Tn5 insect cells (Liu et al. 2008). Insect Sf9 and Tn5 cells were infected with recombinant baculovirus AcPCV2-ORF2 that contains the complete PCV2 capsid protein. As compared to Sf9 insect cells, Tn5 expressed higher amount of PCV2 capsid protein as virus-like particles of size around 28-KDa. This vaccine was commercialized by two different names "Circumvent PCV and Porcilis PCV" in different geographical areas by MSD Animal Health (known as Merck Animal Health in the USA and Canada) (Felberbaum 2015). Vaccine for PCV2-based ORF2 was also commercialized as "Ingelvac CircoFLEX" by Boehringer Ingelheim Vetmedica Inc. (Desrosiers et al. 2009).

Table 9.1 BEVs produced biomolecules as disease preventive agents

Product name (company name, if any)	Targeted/used for	Expressed product	Used against	Product type	Development stage
Porcilis Pesti (MSD Animal Health)	Pigs	E2 glycoprotein	Classical swine fever	Protein subunit/ marker vaccine	Approved
Bayovac CSF E2 (Bayer Biologicals/Pfizer Animal Health)[a]	Pigs	E2 glycoprotein	Classical swine fever	Protein subunit/ marker vaccine	Approved
Circumvent PCV (MSD Animal Health)[b]	Pigs	Porcine circovirus ORF2	Porcine circovirus type 2	VLP vaccine	Approved
Porcilis PCV (MSD Animal Health)[b]	Pigs	Porcine circovirus ORF2	Porcine circovirus type 2	VLP vaccine	Approved
Ingelvac CircoFLEX (Ingelvac)#	Pigs	Porcine circovirus ORF2	Porcine circovirus type 2	VLP vaccine	Approved
AcAs3-PPV-VLP	Pigs	Viral capsid protein VP2	Porcine parvovirus (PPV)	VLP vaccine	Unapproved
BTV-1/BTV-4 VLP	Sheeps	BTV serotype 1 and 4	Bluetongue virus (BTV)	VLP vaccine	Unapproved
AI-H5N3 VLP	Ducks	Subunits HA, NA and M1	Avian influenza (AI)	VLP vaccine	Unapproved
IDBV-VLP	Chickens	Capsid proteins VP2, VP3 and VP4	Infectious bursal disease virus (IBDV)	VLP vaccine	Unapproved
RHDV-VLP	Rabbits	Capsid proteins VP60	Rabbit haemorrhagic disease virus (RHDV)	VLP vaccine	Unapproved
SIV-VLP	Primates	Precursor protein Pr56gag	Simian immunodeficiency virus (SIV)	VLP vaccine	Unapproved
Flublok (Protein Sciences)	Humans	Influenza HA	Trivalent flu vaccine	Protein subunit vaccine	Approved
Cervarix (GlaxoSmithKline)	Humans	Human papillomavirus L1 protein (serotypes 16 and 18)	Cervical cancer	VLP vaccine	Approved
Ebola-VLP	Humans	Ebola VP40 and GP protein	Ebola virus	VLP vaccine	Preclinical

Bac-P1-3CD	Humans	EV71-P1 protein and 3CD protease	Enterovirus 71	VLP vaccine	Preclinical
VAI-VP705 (NIH/Meridian Life Science)	Humans	B19 VP1, VP2	Parvovirus B19	VLP vaccine	Phase I/II
NV-VLP (Baylor College of Medicine)	Humans	Capsid proteins NV CP	Norwalk virus (Nv)	VLP vaccine	Phase I
NV-VLP (Ligo Cyte Pharmaceuticals)	Humans	Capsid proteins NV CP	Norwalk virus (Nv)	VLP vaccine	Phase I
NV-VLP (Ligo Cyte Pharmaceuticals)	Humans	Capsid proteins NV CP	Norwalk virus (Nv)	VLP vaccine	Phase I/Phase I/II
PV-VP1-VLP	Humans	Major capsid protein VP1	Polyomavirus	VLP vaccine	Preclinical
SARS-CoV-VLP	Humans	SP, EP and MP	Severe acute respiratory syndrome-associated coronavirus (SARS-CoV)	VLP vaccine	Preclinical
SV40-VLP	Humans	VP1 or P1 and 3CD	Simian virus 40 (SV40)	VLP vaccine	Preclinical
RV-VLP	Humans	VP2, VP6 and VP7	Rotavirus	VLP vaccine	Preclinical
HIV-VLP	Humans	Gag protein	HIV	VLP vaccine	Preclinical
Influenza (Novavax)	Humans	A/California/04/09(H1N1) HA, NA	Influenza	VLP vaccine	Phase II
Influenza (Novavax)	Humans	A/Brisbane/59/07(H1N1), A/Brisbane/10/07, B/Florida/04/06 (H3N2)	Influenza	VLP vaccine	Phase IIa
RSV (Novavax)	Humans	RSV-F	RSV	VLP vaccine	Phase I

[a] Discontinued

[b] MSD Animal Health got the same products licensed by two names in different geographical areas

(c) *VLP-based vaccine for porcine parvovirus (PPV)*: PPV, a non-enveloped DNA virus, causes major reproductive failure in swine. Its viral capsid is made up of 50–60 molecules of VP2, the major structural protein that are being targeted for vaccine development. VP2 gene was expressed under the control of late p10 promoter of baculovirus and the *LacZ* gene under the control of *Drosophila hsp 70* promoter. The recombinant baculovirus AcAs3-PPV was used to infect Sp21 insect cell line to express VP2 that leads to self-assembled empty PPV VLPs in serum-free medium for safety point of view (Maranga et al. 2002). Earlier, it was also produced in *Sf9* cells in the presence of serum proteins. However, its commercialization at large scale still needs more developmental efforts.

(d) *VLP-based vaccine for sheep bluetongue virus (BTV)*: Bluetongue primarily causes disease in ruminants due to infection by BTV double-stranded RNA virus. Sheep generally shows more severe clinical signs than other cattle. Recombinant baculovirus expression system in *Sf9* insect cell lines shows great potential to develop VLP-based vaccine against BTV (de Diego et al. 2011). Monovalent and bivalent VLP vaccines are being developed for two serotypes 1 and 4 of BTV. BTV-1 exhibits more protection to virulent BTV live strain as compared to BTV-4. Earlier, VLP expressing capsid proteins VP2 and VP5 were developed by co-transfection of dual transfer vector DNA (pAcVC3/BTV-10-2/BTV-10-5) with wild-type AcNPV DNA in insect cells (French et al. 1990). Strong developmental efforts and further research are needed to commercialize robust and effective BTV vaccine.

Many more VLPs veterinary vaccines by baculovirus expression system in insect cells have been developed such as avian influenza (AI), (H5N3)-VLPs that consists of subunits haemagglutinin (HA), neuraminidase (NA) and matrix protein (M1) of AI virus for ducks (Prel et al. 2008); chimeric infectious bursal disease virus (IBDV)-VLPs consisting of structural protein VP2, VP3 and VP4 with varying degree of one of the capsid protein VP2 tagged with histidine of IBDV for chickens (Hu and Bentley 2001); rabbit haemorrhagic disease virus (RHDV)-VLPs made up of VP60 capsid protein for rabbits (Laurent et al. 1994); and simian immunodeficiency virus (SIV)-VLPs consisting of precursor protein $Pr56^{gag}$ for vaccine testing in non-human primates (Yamshchikov et al. 1995).

9.1.1.2 Human Vaccines

(a) *Subunit vaccine for influenza*: Influenza generally called as “the flu” is caused by RNA influenza viruses, designated from type A to C. Both type A and B influenza viruses possess haemagglutinin (HA) or neuraminidase (NA) glycoprotein spikes in their envelope which act as key antigens in the host immune response, therefore targeted for vaccine development. But HA and NA exhibit antigenic drift due to continuous mutations in the genetic material, and the vaccine based on these glycoproteins is required to be updated annually. However, type C influenza virus is not involved in annual influenza virus vaccine as they cause only mild respiratory disease in humans. The vaccine against influenza in *Spodoptera frugiperda Sf9* insect cells is developed by targeting dominant

surface glycoproteins HA of influenza virus. Recombinant viruses as vaccines are produced in Sf9 insect cells by co-transfecting linearized AcMNPV genomic DNA with the baculovirus transfer plasmids containing the HA gene. Recombinant plaques are selected on the basis of their morphology and virus stocks generated. These viral stocks are used for their amplification through passage in the fresh insect cell cultures. Commercial production of recombinant HA vaccine could begin within 45 days after identification of the new virus strain. It is commercialized by Protein Sciences Corporation by Flublok trademark (Cox and Hashimoto 2011).

(b) *VLP-based vaccine for human papillomavirus (HPV)*: HPV infection causes mostly all forms of cervical cancer in women. HPVs are icosahedral viruses with double-stranded circular DNA codes for two classes of genes; early (E) and late (L). Early genes regulate replication, transcription and other biological processes, whereas late genes (L1 and L2) are responsible for structural components of the viral capsid. L1 capsid proteins are known to form virus-like particles, therefore targeted for vaccine development against HPV. L1-based vaccine in insect cells that shows remarkable safety profile and clinical efficacy from the genotypes HPV16 and 18 was commercialized by GlaxoSmithKline by the trademark Cervarix (Monie et al. 2008). It has been produced in *Trichoplusia ni* insect cell lines Hi-5 Rix4446 by using baculovirus expression system.

With the advent of successful cases of approved VLP-based vaccines, researchers are indeed redirecting their efforts for the development of such products. Therefore, a number of vaccines have been produced against many viral diseases in humans; however many of them are either in preclinical or clinical trial stages. Prominent VLPs that are made up of multimeric proteins expressed in insect *Sf9* cells include Ebola by VP40 and glycoproteins (Sun et al. 2009); enterovirus by P1 and 3CD (Chung et al. 2010); human parvovirus B19 by B19 VP1, VP2 (Roldão et al. 2010); Norwalk virus (Nv) by capsid proteins (Jiang et al. 1992; Ball et al. 1999; Atmar et al. 2011; Frey 2011); polyomavirus by VP1 (Montross et al. 1991); severe acute respiratory syndrome-associated coronavirus (SARS-CoV) by SP, EP, MP and EN (Mortola and Roy 2004) and simian virus 40 (SV40) by VP1 or P1 and 3CD (Kanesashi et al. 2003). VLPs for rotavirus were prepared by using two (VP2 and VP6) to three (VP2, VP6 and VP7) capsid proteins expressed both in *Sf9* and High Five insect cells. It has also been expressed in *Sf larvae* with two capsid proteins VP2 and VP6 (Roldão et al. 2010). Combinations of capsid proteins from different strains of influenza were used in both *Sf9* and High Five insect cells such as HA (H1N1) with M1 (H3N2) and HA (H3N2) with M1 (H1N1) to produce higher amount of influenza A-VLPs. Other influenza A-VLPs formed by co-expression of M1 and ESAT6-HA were produced only in High Five cells. Strain-specific influenza HA and M1 capsid proteins were used to prepare influenza A H1N1-VLPs and influenza A H3N2-VLPs in both the insect cells (Krammer et al. 2010; López-Macías et al. 2011). Respiratory syncytial virus (RSV) vaccine was produced by using RSV-F protein (Mazur et al. 2015; Neuzil 2016). HIV VLPs were produced by targeting gag protein in rodents and rhesus macaques for preclinical trials (Pillay et al. 2009; Wagner et al. 1996).

9.1.2 BEVs as Diagnostic Agents

Supposedly, both heterologous subunit proteins and VLP-based subunit vaccines can be used as vaccines as well as antigens for the detection of antibodies, given the condition that it satisfies the various diagnostic parameters like sensitivity, specificity, predictive values and likelihood ratios. These parameters have been well evaluated and found to be acceptable for diagnostic purposes for numerous BEV-derived products. However, commercialization of these vaccines/proteins demands further standardization and evaluation. Here, we have summarized some of the human as well as veterinary usage diagnostic molecules produced by BEV system in insect cells (Table 9.2).

Table 9.2 BEVs produced biomolecules as diagnostic agents

Targeted/used for	Expressed product	Used to detect	Test type
Rodents	Recombinant nucleocapsid protein	Sendai virus	ELISA
Swine	G-protein	Nipah virus (NiV)	ELISA
Swine	SVDV-VLP	Swine vesicular diseases virus (SVDV)	ELISA
Horse	EIAV-core gag and p26 protein	Equine infectious anaemia virus (EIAV)	ELISA and agar gel immunodiffusion (AGID)
Horse	HA	Equine influenza strain LP/93	ELISA
Cattles	VP7	Bluetongue (BTV) and epizootic haemorrhagic disease (EHDV)	Antigen capture competitive ELISA (Ag Cap c-ELISA)
Pigs and goats	VP1 capsid protein and 3C protease	Foot-and-mouth disease virus (FMDV)-type A	Blocking ELISA
Bovine	Recombinant-F protein	Bovine respiratory syncytial virus (BRSV)	Immunofluorescence
Ducks	E protein	Tembusu virus (TMUV)	E-ELISA
Birds	APMV2-HN	Avian paramyxovirus type 2	Haemagglutination inhibition (HI) test
Geese	VP1	Goose haemorrhagic polyomavirus (GHPV)	ELISA and haemagglutinin inhibition test
Humans	Nucleocapsid protein (N)	Lassa virus	ELISA
Humans	E1, E2 and polyprotein precursor	Rubella virus (RV)	Enzyme immunoassay (EIA) and immunoblot
Humans	Flagellar repetitive antigen (FRA)	*Trypanosoma cruzi*	ELISA

Table 9.2 (continued)

Targeted/used for	Expressed product	Used to detect	Test type
Humans	Glutamic acid decarboxylase (GAD65 and GAD67)	Insulin-dependent diabetes mellitus	Immunoassay
Humans	Nucleocapsid protein of strain SR-11	Hantavirus	Indirect immunofluorescence antibody (IFA)
Humans	E2 protein	Human papillomavirus (HPV)	ELISA
Humans	Hou/90 capsid	Human calicivirus (HuCV)	Immunoprecipitation and EIA
Humans	Fragment of gG comprising residues 321–580 of HSV-2	Herpes simplex virus (HSV)	Indirect ELISA
Humans	Capsid proteins	Human caliciviruses (HuCVs)	EIA
Humans	C-terminus truncated form of protein (Etr)	TBE complex virus	ELISA and immunoblot assay
Humans	Recombinant Fel dl (rFel dl Ch1 + Ch2)	Cat allergen	Radioimmunoassay (RIA) and ELISA
Humans	Recombinant human tissue TG (hu-tTG)	Autoantigen transglutaminase (TG)	ELISA
Humans	Envelop glycoproteins gB, gD, gC, gE and gG	Herpes B virus (HBV)	ELISA

9.1.2.1 Veterinary Applications

(a) *Rodent*: Recombinant nucleocapsid protein produced in baculovirus expression system-based enzyme-linked immunosorbent assay (ELISA) is reported to be more specific as compared to whole virion conventional ELISA for the detection of Sendai virus infection in rodents (Wan et al. 1995).

(b) *Swine*: Specific indirect ELISA method was developed for the detection of Nipah virus (NiV) infection in swine serum samples by cloning G-protein of NiV into pFASTBac HT vector (Eshaghi et al. 2004). Its further use as diagnostic reagent for humans needs to be explored. P1 and 3CD protein genes of swine vesicular disease virus (SVDV)-derived VLPs as antigens for detection of antibodies against SVDV in pigs by ELISA were also developed (Ko et al. 2005).

(c) *Horse*: Recombinant baculovirus expressing equine infectious anaemia virus (EIAV) core proteins Gag and p26 as antigens was found to possess high specificity and sensitivity in ELISA and agar gel immunodiffusion (AGID) to detect antibodies from infected horse sera (Kong et al. 1997). Haemagglutinin (HA)

protein of equine influenza strain, A/equine/La Plate/I/93 (LP/93), was produced in silkworm larvae with recombinant baculovirus for the detection of antibodies in horse sera by ELISA (Sugiura et al. 2001). Its efficiency was further tested by HA1 subunit of HA (Sguazza et al. 2013).

(d) *Cattle*: Baculovirus-derived antigen capture competitive ELISA (Ag Cap c-ELISA) for the diagnosis of bluetongue and epizootic haemorrhagic disease virus infection in cattle exhibits advantages in terms of easy production, standardization, less requirement of downstream processing and its non-infectious nature as compared to commercially available c-ELISA (Mecham and Wilson 2004). Blocking ELISA was developed by BEVs for the detection of antibodies against foot-and-mouth disease of cattle, pigs and goats by virus type A with a specificity of 99% (Ko et al. 2010). Bovine respiratory syncytial virus (BRSV) infection that causes lower respiratory tract disease in calves 1–3 months old can be detected by immunofluorescence analysis with recombinant F-protein as antigen (Pastey and Samal 1998).

(e) *Bird*: A variant of ELISA known as E-ELISA using eukaryotically expressed E protein as the antigen for the detection of Tembusu virus (TMUV) in ducks was developed with 93.2% specificity and 97.8% sensitivity (Yin et al. 2013). Recombinant avian paramyxovirus type 2 haemagglutinin (APMV2-HN) is found to be a useful alternative to APMV-2 antigens in haemagglutination inhibition (HI) test for the detection of APMV-2 infection in avians (Choi et al. 2014). Whole Sendai virus virion VLPs are being used as antigens for the detection of antibodies against virus for diagnostic purposes such as major capsid protein VP1 of goose haemorrhagic polyomavirus-VLPs for the detection of GHPV-specific antibodies in sera from flocks with haemorrhagic nephritis and enteritis of geese (HNEG) disease (Zielonka et al. 2006).

9.1.2.2 Application in Humans

Most of the recombinant proteins that are used as antigens have been expressed by baculovirus expression system in *Sf9* insect cells unless otherwise stated. Some of them are mentioned here.

Lassa virus infection causes Lassa fever mainly endemic in West Africa. Recombinant nucleocapsid protein acts as antigen for the detection of antibodies in Lassa virus-infected patient sera by ELISA (Barber et al. 1990; Saijo et al. 2007). Rubella virus (RV) normally causes a self-limiting disease, but its infection during the first trimester of pregnancy may cause foetal damage. Therefore, serological diagnostic test was developed by expressing E1, E2 and polyprotein precursor of rubella virus as antigen for enzyme immunoassay (EIA) and immunoblot analysis of patient sera (Seppänen et al. 1991). *Trypanosoma cruzi* causes Chagas' disease in Latin America. Flagellar repetitive antigen (FRA), part of *T. cruzi*-based improved diagnostic assay, was developed for Chagas' disease (dos Santos et al. 1992). Full-length human glutamic acid decarboxylases (GAD65 and GAD67) with histidine tag were produced in their natural conformations for the development of an immunoassay for the diagnosis of insulin-dependent diabetes mellitus (Mauch et al. 1993). Hantavirus which causes haemorrhagic fever with renal syndrome (HFRS)

nucleocapsid protein of strain SR-11 (rNP-SR-Sf9) was used as antigen for the indirect immunofluorescence antibody (IFA) diagnostic test that detects three serotypes (hantan 76-118, SR-11 and Puumala) of hantavirus (Yoshimatsu et al. 1993). Purified human papillomavirus (HPV) E2 protein was used to develop ELISA to detect IgG and IgA responses in cervical neoplasia patients (Rocha-Zavaleta et al. 1997). Houston/90 (Hou/90) is a human calicivirus (HuCV) strain in one of the three clades of Sapporo-like HuCVs that cause acute gastroenteritis in children. The viral capsid gene of Hou/90 capsid was used as antigen for immunoprecipitation and EIA (Jiang et al. 1998). Herpes simplex virus (HSV) infection is caused by two viruses HSV-1 and HSV-2. Diagnostic test that can distinguish between two strains has been developed that utilizes both type-specific and type-common HSV antigens in a single-step assay format to perform accurate diagnosis (Burke 1999; Wald and Ashley-Morrow 2002; Liu et al. 2015). Eight different strains of human caliciviruses (HuCVs) capsid proteins have been used to develop antigen-antibody detection assay by EIAs that are highly specific (Jiang et al. 2000). Causative agent of tick-borne encephalitis (TBE), C-terminus truncated form of protein E (Etr) of TBE complex virus tagged with histidine was used to develop sensitive and specific ELISA as well as immunoblot assay to detect the TBE virus-specific antibodies in infected individuals (Marx et al. 2001). Fel dl, the major allergen from cats, consists of two polypeptide chains, chain 1 (ch1) and chain 2 (ch2), which are usually linked with a disulphide bond. Recombinant Fel dl (rFel dl Ch1 + Ch2) protein construct in which two chains are linked together with glycine/serine linker was used as more potent antigen than bacterial-derived proteins for the detection of IgE and IgG antibodies by radioimmunoassay (RIA) and ELISA (Guyre et al. 2002). Coeliac disease (CD) is characterized by the presence of autoantigen transglutaminase (TG). Recombinant human tissue TG (hu-tTG) expressed with baculovirus system was used as antigen for ELISA that showed a sensitivity of 100% and a specificity of 98.6% (Osman et al. 2002). The envelope glycoproteins: gB, gD, gC, gE and gG are thought to be the primary targets of IgG antibody response in patients with Herpes B virus (HBV) infection. Therefore, ELISA test was developed by using the cocktail of these recombinant glycoproteins along with other capsid proteins with high sensitivity and specificity (Perelygina et al. 2005). Similarly, the recombinant proteins in single or multiple subunits for the diagnosis of different types of viral infections in humans have been developed with baculovirus expression system in insect cells.

9.1.3 BEVs as Therapeutic Agents

BEVs express products like growth factors, cytokines, chemokines, enzymes, hormones and monoclonal antibodies that can be used for human therapeutic purposes. More recently, BEV has also been exploited as effective tool for gene therapy. For simplicity, the applications of these products have been divided into two major groups: biological drug therapy and gene therapy. Over thousands of such biomolecules have been developed till now in this system, few among them are discussed here (Table 9.3).

Table 9.3 BEVs produced biomolecules as disease therapeutic agents

Therapy type	Targeted for	Product type	Product name	Expressed product	Development stage	Company name, if any
Immunotherapy	Prostate cancer	Recombinant fusion protein	Provenge or sipuleucel-T	PAP-GM-CSF^	Approved	Dendreon
Immunotherapy	Colorectal carcinoma	Monoclonal antibodies	Anti-GA733-2E	CO17-1A Mab (IgG2a)	Unapproved	
Immunotherapy	Haematolymphoid cells	Recombinant protein	Anti-Bcl-2-Mab	B-cell lymphoma leukaemia-2 (Bcl-2) protein	Unapproved	
Immunotherapy	Rotavirus	Single-domain antibodies (sdAbs)	3B2 and 2KD1 antibodies	Anti-VP6	Unapproved	
Immunotherapy	Breast cancer	Monoclonal antibodies	mAb-BR55/mAb-BR55K	HC and LC	Unapproved	
Immunotherapy	Antigen-presenting cells (APCs)	Adjuvant antibody	APCH1 antibody	Anti-MHC class II DR	Unapproved	
Immunotherapy	Immune cells	Cytokine	IL-2	Human interleukin 2	Unapproved	
Immunotherapy	Stem cells, macrophages	Cytokine	hGM-CSF	Human granulocyte-macrophages colony-stimulating factor	Unapproved	
Enzyme therapy	Purine salvage pathway	Enzyme	ADA	Human adenosine deaminase	Unapproved	

Hormone therapy	Hypoparathyroidism	Hormone	hPTH	Human parathyroid hormone	Unapproved	
Growth factor therapy	Wound healing	Growth factor	huEGF1	Human epidermal growth factor	Unapproved	
Growth factor therapy		Growth factor	huFGF2	Human fibroblast growth factor 2	Unapproved	
Growth factor therapy		Growth factor	huKGF1	Human keratinocyte growth factor 1	Unapproved	
Growth factor therapy	Alzheimer's disease	Growth factor	rhNGF	Human prepro (beta) nerve growth factor	Unapproved	
Enzyme-gene therapy	Familial lipoprotein lipase deficiency	Transgene	Glybera or LPL^{S447} × transgene	Lipoprotein lipase transgene	Approved	UniQure
Protein gene therapy	Haemophilia A	Transgene	AAV-FVIII	Factor VIII	Unapproved	
Protein gene therapy	Haemophilia B	Transgene	AAV8-hFIX19	Factor IX	Phase I	Spark Therapeutics
			AskBio009 (AAV8)		Phase I/II	Baxalta US Inc.
			scAAV 2/8-LP1-hFIXco		Phase I	St. Jude Children's Research Hospital
			AAV2-hFIX16		Phase I	Spark Therapeutics
Enzyme-gene therapy	Leber congenital amaurosis	Transgene	AAV2-hRPE65v2	Retinoid isomerohydrolase	Phase III	Spark Therapeutics
			rAAV2-CB-hRPE65		Phase I/II	Applied Genetic Technologies Corp

(continued)

Table 9.3 (continued)

Therapy type	Targeted for	Product type	Product name	Expressed product	Development stage	Company name, if any
			tgAAG76 (rAAV 2/2.hRPE65p.hRPE65)		Phase I/II	University College, London
			rAAV2/4.hRPE65		Phase I/II	Nantes University Hospital
			rAAV2-CBSB-hRPE65		Phase I	University of Pennsylvania
			rAAV2-hRPE65		Phase I	Hadassah Medical Organization
Enzyme-gene therapy	Leber's hereditary optic neuropathy	Transgene	AAV2-ND4	NADH-ubiquinone oxidoreductase chain 4	Phase I	John Guy, University of Miami
Enzyme-gene therapy	Age-related macular degeneration	Transgene	AAV2-soluble Flt1	Soluble fms-like tyrosine kinase	Phase I	Genzyme, a Sanofi Company
Enzyme-gene therapy	Canavan disease	Transgene	AAV-ASAP	Aspartoacylase	Unapproved	
Growth factor-gene therapy	Alzheimer's disease	Transgene	AAV-NGF or CERE-110	Beta-nerve growth factor	Phase I	Sangamo Therapeutics (Ceregene)
Enzyme-gene therapy	Parkinson's disease	Transgene	AAV2-GAD	Glutamic acid decarboxylase	Phase II	Neurologix, Inc.
Protein gene therapy			AAV2-NTN or CERE-120	Neurturin	Phase II	Ceregene
Enzyme-gene therapy			AAV-hAADC-2	Aromatic L-amino acid decarboxylase	Phase I	Genzyme, a Sanofi Company
			AAV2-hAADC		Phase I	Voyager Therapeutics

Protein gene therapy			AAV2-GDNF	Glial cell line-derived neurotrophic factor	Phase I	National Institutes of Health Clinical Center (CC)
Protein gene therapy	Duchenne muscular dystrophy	Transgene	rAAV2.5-CMV-minidystrophin	Mini-dystrophin	Phase I	Asklepios BioPharmaceutical, Inc.
Protein gene therapy	Becker muscular dystrophy	Transgene	rAAV1.CMV.huFollistatin344	Follistatin	Phase I	Nationwide Children's Hospital
Protein gene therapy	Limb girdle muscular dystrophy	Transgene	AAV1-gamma-sarcoglycan	Gamma-sarcoglycan	Phase I	Genethon
Protein gene therapy	Spinal muscular atrophy	Transgene	scAAV9.CB.SMN	Survival motor neuron	Phase I	AveXis, Inc.
Enzyme-gene therapy	Acute intermittent porphyria	Transgene	rAAV2/5-PBGD	Porphobilinogen deaminase	Phase I	Digna Biotech S.L.
Enzyme-gene therapy	Alpha 1-antitrypsin deficiency	Transgene	rAAV1-CBhAAT	Alpha 1-antitrypsin	Phase II	Applied Genetic Technologies Corp
Enzyme-gene therapy			rAAV2-CBhAAT		Phase I	University of Massachusetts, Worcester
Enzyme-gene therapy			AAVrh.10halpha1AT		Phase I	Adverum Biotechnologies, Inc.
Enzyme-gene therapy	Aromatic amino acid decarboxylase deficiency	Transgene	AAV2-hAADC	Aromatic L-amino acid decarboxylase	Phase I/II	National Taiwan University Hospital
Protein gene therapy	Choroideremia	Transgene	rAAV2.REP1	Rab-escort protein 1	Phase I	University of Oxford

(continued)

Table 9.3 (continued)

Therapy type	Targeted for	Product type	Product name	Expressed product	Development stage	Company name, if any
Enzyme-gene therapy	Chronic heart failure	Transgene	AAV1-CMV-SERCA2a	Sarcoplasmic reticulum calcium ATPase	Phase II	Imperial College London, Assistance Publique—Hôpitaux de Paris and Celladon Corporation
Protein gene therapy	Gastric cancer	Transgene	AAV-DC-CTL	Carcinoembryonic antigen	Phase I	Tianjin Medical University Cancer Institute and Hospital
Enzyme-gene therapy	HIV	Transgene	AAV-2 HIV vaccine (tgAAC09)	Gag, protease and reverse transcriptase parts	Phase I	International AIDS Vaccine Initiative
Antibody-gene therapy			rAAV1-PG9DP	PG9 antibody	Phase I	International AIDS Vaccine Initiative
Receptor-gene therapy	Inflammatory arthritis	Transgene	tgAAC94	TNFR:Fc fusion gene	Phase 1/II	Targeted Genetics Corporation
Protein gene therapy	Late infantile neuronal ceroid lipofuscinosis	Transgene	AAVrh.10CUCLN2	Neuronal ceroid-lipofuscinosis 2	Phase 1/II	Weill Medical College of Cornell University
Protein gene therapy			AAV2CUhCLN2	Neuronal ceroid-lipofuscinosis 2	Phase I	Weill Medical College of Cornell University
Trinucleotide-gene therapy	Pompe disease	Transgene	rAAV1-CMV-GAA	Normal GAA	Phase 1/II	University of Florida

9.1.3.1 Biological Drug Therapy

BEVs have been utilized as eukaryotic expression vectors in insect cells for the production of therapeutic or immunotherapeutic proteins such as monoclonal antibodies, cytokines and chemokines, growth factors, etc. that require post-translational modifications, more importantly glycosylation. The baculovirus expression system has been accepted as one of the most efficient and powerful technologies for the production of biological recombinants in terms of achievable quantity, purity and ease of the eukaryotic processing (Luckow and Summers 1988). Therapeutic recombinant protein production is considered as an essential section of the emerging biotechnology industries. This system has the potential for the development of high commercial value industry.

(a) *Immunotherapy*: Over the years, numerous tumour immunotherapies achieved early-stage successes but failed in clinical trials Phase-III (Goldman and DeFrancesco 2009). Baculovirus-derived Dendreon's Provenge (Seattle; sipuleucel-T) for prostate cancer is among the first therapeutic cancer vaccines to complete Phase-III trail successfully and to receive FDA approval. Provenge (Sipuleucel-T) is an autologous active cellular immunotherapy that has shown evidence of reducing the risk of death among men with metastatic castration-resistant prostate cancer (Kantoff et al. 2010). It consists of autologous peripheral-blood mononuclear cells (PBMCs), including antigen-presenting cells (APCs), which have been activated ex vivo with a recombinant fusion protein (PA2024). The fusion protein PA2024 contains prostate antigen, prostatic acid phosphatase which is fused to an immune-cell activator called granulocyte-macrophage colony-stimulating factor. PA2024 is produced by BEV in *Sf21* insect cells.

Monoclonal antibody CO17-1A was prepared against colorectal cancer cells by using pFastBac vectors (Park et al. 2011). The BEVs expressed proteins that are being utilized for the production of monoclonal antibodies against Bcl-2 (B-cell lymphoma leukaemia-2). It is an integral membrane oncoprotein that regulates programmed cell death (apoptosis) in haematolymphoid cells (Reed et al. 1992). Single-domain antibodies (sdAbs) that are prepared against rotavirus infection are also known as nanobodies or VHHs. They have characteristically high stability, solubility and very high affinity for their antigens. These antibodies were first produced in the insect larvae *Trichoplusia ni* which serve as living bio-factories for the production of these biomolecules (Gómez-Sebastián et al. 2012). Anti-breast cancer monoclonal antibodies (mAb) BR55, with or without fusion with KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum retention signal, were prepared. The heavy chain (HC) and light chain (LC) genes of mAb BR55 were cloned in pFastBac Dual vector under the control of polyhedrin (P_{PH}) and p_{10} promoters, respectively, in Sf9 insect cells (Lee et al. 2014). Antibody response was enhanced against two recombinant subunit vaccines by tagging the vaccines with adjuvant recombinant single-chain antibody APCH1. It recognizes the MHC Class II DR and produced in *Trichoplusia ni* insect cells (Gil et al. 2011).

Human interleukin 2 (IL-2) was prepared in insect larvae of *T. ni* by placing the IL-2 gene under p10 promoter of BEV (Pham et al. 1999). Human granulocyte-macrophages colony-stimulating factor (hGM-CSF) was prepared in *Bombyx mori* (silkworm) nuclear polyhedrosis virus (BmNPV) (Shi et al. 1996). Other cytokines and chemokines are being produced by using this expression system in a similar manner.

Recently, the intravesical instillation of transgene devoid baculovirus is found to elicit local immune stimulation by upregulating a set of Th-1-type cytokines in orthotopic bladder tumours in mice (Ang et al. 2016). However, the application of such strategy for non-muscle invasive bladder cancer (NMIBC) in humans is awaited.

(b) *Enzyme and hormonal therapy*: Enzyme human adenosine deaminase, a key purine salvage enzyme required for immune competence, has been produced both in *Trichoplusia ni* and *Spodoptera frugiperda* insect cells as well as larvae. This enzyme possessed specific activity of 70 units/mg in crude homogenate that is 70–350 times higher than its two most abundant natural sources thymus and leukemic cells. Such biologically active, inexpensive, rapid and huge production of the enzymes by this baculovirus system opens up the avenues for other biologically active molecules. Human parathyroid hormone (hPTH) was produced both in *Bombyx mori* cells and larvae. Both of the host systems have been reported to be suitable for efficient synthesis and secretion of the correctly processed hPTH (Mathavan et al. 1995). Similarly, recombinant full length human growth hormone (hGH) was produced in *Bombyx mori* nuclear polyhedrosis virus (vBmhGH) (Sumathy et al. 1996).

(c) *Growth factors therapy*: Growth factors are naturally signalling molecules required for myriads of biological processes for which the requirement of consistent, cost-effective and clinically efficient technologies is indispensable. Wound healing is one of such complex biological processes that requires the collaborative efforts of different tissues, cells and molecules. The repair process of wounds after injury is initiated by the release of various growth factors (GFs). GFs act as functional messenger molecules between cells which control the cellular processes in the regulatory network and sometimes require recombinant protein therapies. Currently, wound healing is being focussed on GFs and/or human skin substitutes, required for decreasing healing time by modifying inflammation and accelerating the proliferative phase. The beneficial effects of GFs to attract different kinds of cells at the site of wound healing have been demonstrated by many studies. Wider clinical and commercial applications of such GFs depend on their scalable cost-efficient production. BEVs have been successful in unblocking the bottlenecks for such inevitabilities. Three fully functional human GFs, the human epidermal growth factor 1 (huEGF1), the human fibroblast growth factor 2 (huFGF2) and the human keratinocyte growth factor 1 (huKGF1), have been produced with BEVs in *Trichoplusia ni* insect larvae (Dudognon et al. 2014). The expression of huKGF1 was found to be enhanced further when it was expressed by tagging it with human antibody IgG fragment crystallisable region (Fc).

Human prepro (beta) nerve growth factor that has been suggested as a therapeutic agent for the treatment of Alzheimer's disease was produced in insect cells as recombinant virus, mature human beta nerve growth factor (rhNGF). It was found to be biologically active in cholinergic cell survival (Barnett et al. 1990). Similarly, different strategies are being worked out with BEVs in insect cells or larvae for biologically active, cost-effective, therapeutic and commercial scale production of numerous highly variable molecules.

9.1.3.2 Gene Therapy

Today, gene therapy potential has reached to the point whereby it can be exploited to treat many diseases that were earlier thought to be untreatable. The requisite modalities for such gene drugs such as safety, generation, immune response, duration of expression and the gene delivery capacity are being successfully realized by baculovirus-based vectors. Baculoviruses have been found to deliver genes into a wide range of vertebrate cells and species. However, the exact mechanism of entry of baculovirus in to the host cells is still not fully understood. Recently, phagocytic-like mechanism of entry into mammalian cells was found to be more convincing than pinocytosis (Long et al. 2006). Baculovirus progeny production occurs in two forms, budded virus (BuV) and occlusion-derived virus that only differ in their envelops. BuV derives its envelop from cell membrane and spreads the infection within host, whereas occlusion-derived virus envelop is derived from nuclear membrane and spreads infection between hosts. BuV is the most widely used form in biotechnology that enters the insect and other hosts through endocytosis mechanism, although the tenet of exact endocytic mechanism still needs to be build.

AcMNPV is the prototype of baculoviruses and widely used for different applications including gene therapy. It is able to transduce both dividing and non-dividing mammalian cells and activates the transgene in the target cells that it carries under the control of specified promoter. It indicates that the nucleocapsid of the baculovirus transports its genome across the intact host cell nuclear membrane through nuclear pore complex. However, the detailed molecular mechanism of baculovirus transduction in mammalian cells demands further investigation for efficient gene delivery. BEVs gene delivery capability have been exploited in understanding the mechanism of vertebrate cell transduction, preclinical studies, vaccination, cartilage and bone tissue engineering, cancer gene therapy, assay development, drug screening and generation of other gene therapy vectors (Airenne et al. 2013). We would like to emphasize on the use of recombinant adeno-associated vectors (rAAV) as gene therapy tools which are highly important from bioprocess and therapeutic perspective.

BEVs-Derived Recombinant Adeno-Associated Viruses (rAAVs) for Gene Therapy Recombinant AAVs that carry therapeutic DNA turn out to be the attractive gene delivery vectors because of their suitability for in vivo gene therapy potential, relatively long-term gene expression, lack of pathogenicity and ability to transduce wide variety of both dividing and non-dividing cells. Nine different serotypes of rAAVs are used for gene therapy whereby each serotype exhibits different

propensity for tissue-specific infection and infection kinetics (Zincarelli et al. 2008). The major limitation of low production quantity was addressed recently by optimizing the BEVs platforms and adjusting different parameters such as multiplicity of infection, cell density and fermentation mode that produced up to 10^4 vector genomes per litre (Mena et al. 2010).

The strategy for rAAV production requires the production of three AAV capsid proteins, VP1, VP2 and VP3. These capsid proteins assemble within BEV-transduced insect cells to produce icosahedral VLPs (Aucoin et al. 2007). More efficient rAAVs require co-infection of insect cells with three different kinds of baculoviral vectors. The first one is Bac-Rep, expressing the major AAV replication enzymes Rep 78 and Rep 52 essential for AAV genomic replication and packaging, respectively. Second is Bac-Cap, expressing the AAV virion capsid proteins (VP1, VP2 and VP3), and third is Bac-GOI, expressing the gene of interest flanked by AAV inverted terminal repeat elements required for the rescue, replication and packaging of the heterologous gene. Co-infection with these three vectors in insect cells produces efficiently replicated and encapsulated single-stranded AAV vector genome (Weyer and Possee 1991). Further enhancement of AAV in terms of stability, robustness, scalability and high-titre production involves both Rep and Cap protein expression from a single baculovirus (Bac-Rep Cap), i.e. expression of both Rep 78 and Rep 52 transcription from a single mRNA and genetic modifications of the original Bac-Rep and Bac-Cap constructs (Virag et al. 2009). The development of such robust gene delivery vehicles was based on the fact that AAV genome is efficiently replicated in *Sf9* and *Sf21* insect cell lines in a Rep-dependent fashion. Some of the diseases that are being targeted by gene therapy using rAAV are discussed below:

(a) *Gene therapy against lipoprotein lipase deficiency (LPLD)*: It is a rare autosomal recessive genetic and metabolic disorder in which inactivation of familial lipoprotein lipase enzyme occurs due to mutation in gene LPL. Functional lipase is required for plasma triglyceride hydrolysis under normal condition. Inactivated enzyme results into hypertriglyceridemia characterized by frequent abdominal pain and fatty deposits in the skin and retina that in severe cases can lead to fatal pancreatitis, diabetes and onset of cardiovascular diseases. Earlier therapies targeted to lower the plasma triglycerides have not been proved much effective. Alipogene tiparvovec (also known as AAV1-LPL^{S447X} in the early phases of clinical trial) is the first adeno-associated virus (AAV)-mediated gene therapy manufactured by UniQure that got market authorization and government approval in Europe. It is an AAV1 (serotype 1) vector expressing naturally occurring variants of LPL transgene, LPL^{S447X} linked with improved lipid profile and is commercialized by the name of Glybera (Gaudet et al. 2010). It is injected through intramuscular route in the patients that results in natural gain of function of LPL gene variants to muscle tissues. Glybera use significantly lowers plasma triglycerides by increasing the lipoprotein lipase enzyme activity.

The major concern for using such vector-based gene therapy is to prevent both humoral and cell-mediated immune response elicited against viral capsid

proteins that may impact the efficacy and safety of these drugs. Intramuscular injections of Glybera has been proved clinically safe and efficient drug that does not elicit any additional systemic and local immune response harmful for humans. This approach was found to be relevant and promising for the treatment of thousands of single gene disorders. Similar strategies are being investigated in diverse range of therapeutic areas, and many products for the treatment of human diseases are in different stages of clinical development. These AAV gene therapy drugs at different clinical development phases are being discussed here.

(b) *Haemophilia*: It is a blood clotting disorder caused by the mutation in the clotting factor IX gene. Presently, four clinical trials are going on that involve rAAV serotype 2 or 8, designed to express factor IX.

Haemophilia A, the most common severe inherited bleeding disorder caused by mutation in factor VIII gene, is significantly more problematic for this treatment because of a larger size of cDNA that prevents in achieving the adequate level of transgene expression and elicits the anti-factor VIII immune response (High et al. 2014).

(c) *Retinal degeneration*: Recombinant AAV has been used to treat a number of animal models but is limited by carrying capacity, slow onset of expression and limited ability to transduce some of the retinal cell types from the vitreous. Next-generation AAVs have been produced to address these issues by creation of self-complementary AAV vectors for faster onset of expression and specific mutations of self-exposed residues to increase transduction. Such vectors were further improved for broader applicability and advantageous characteristics by directed evolution through an iterative process of selection (Day et al. 2014). Age-related macular degeneration (AMD) that leads to the central vision loss in elderly individuals due to choroidal neovascularization is marked by proliferation of blood vessels and retinal pigment epithelial (RPE) cells. It leads to photoreceptor death and fibrous disciform scar formation. Treatment of AMD patients requires neutralization of vascular endothelial growth factor (VEGF) for which expression of modified soluble Flt1 receptor was designed and expressed in AAV2-sFLT01 vector. Presently, this study is in Phase 1 trial (MacLachlan et al. 2011). Leber congenital amaurosis (LCA) is an autosomal recessive blinding disease that occurs due to mutations in RPE65 gene. Subretinal administration of AAV2-hRPE65v2 has been reported both safe and efficient for at least 1.5 years after injection. Currently six clinical trials, either in stage 1 or 2, are going on to treat this retinal disease (Simonelli et al. 2010).

(d) *Neurological diseases*: rAAV has been used as an effective gene delivery system for the treatment of central or peripheral nervous system with almost no adverse effects in many clinical trials. First time, its clinical use in the human brain has been used to treat Canavan disease, a childhood leukodystrophy also known as Van Bogaert-Bertrand disease caused by the deficiency of enzyme aspartoacylase (ASAP). It involves neurosurgical administration of approximately 10 billion infectious particles of recombinant adeno-associated virus (AAV) containing the aspartoacylase gene (ASPA) directly to the affected

regions of the brain (Janson et al. 2002). To treat Alzheimer's disease, transfer of gene encoding nerve growth factor (NGF), which is essential for healthier nerve cells, is transduced by an adeno-associated nerve growth factor (CERE-110) (Bakay et al. 2007). Transduction of glutamic acid decarboxylase (GAD) and trophic factor neurturin was assessed successfully in different Phase 1 and 2 clinical trials for the treatment of Parkinson's disease (Marks et al. 2010; Kaplitt et al. 2007).

(e) *Duchenne muscular dystrophy (DMD)*: DMD is a severe recessive X-linked muscle disorder caused by mutations in gene encoding dystrophin. Gene therapy to treat DMD is a challenge due to the large size of DMD gene. However, alternative gene delivery strategies like exon skipping, trans-splicing, micro- and mini-dystrophin in Phase II/III clinical trials have been found to be promising (Jarmin et al. 2014).

A number of Phase I/II/III clinical trials are underway for the treatment of numerous diseases such as acute intermittent porphyria, alpha 1-antitrypsin deficiency, aromatic amino acid decarboxylase deficiency, Becker muscular dystrophy, choroideremia, chronic heart failure, gastric cancer, HIV, inflammatory arthritis, late infantile neuronal ceroid lipofuscinosis, Leber's hereditary optic neuropathy, limb girdle muscular dystrophy, macular degeneration, Pompe disease, spinal muscular atrophy, etc. (Felberbaum 2015).

The future prospectives of baculovirus gene delivery applications in stem cell transduction, cancer gene therapy and cartilage and bone tissue engineering are also quite optimistic. Great interest in regenerative medicine begins with the advancement in identification, isolation and derivation of human stem cells, specifically the generation of human-induced pluripotent stem cells. Prolonged expression of transgenes has been demonstrated in multiple multipotent stem cells such as mesenchymal, neural, umbilical cord, bone marrow, adipose tissue, human embryonic stem cells (hESCs) and pluripotent stem cells. These baculoviruses have also been customized for stable gene expression in stem cells by genomic integration for downstream therapeutic applications, for example, deriving unlimited numbers of genetically corrected functional adult cells for cell replacement therapy (Kotin et al. 1991).

De-differentiated chondrocytes transduced with baculovirus vector (Bac-CB) expressing bone morphogenetic protein-2 (BMP-2) result into sustained expression of BMP-2 with passaged chondrocytes in vitro. It was further improved by co-expression of transforming growth factor beta with baculovirus vectors (Chen et al. 2008). These chondrocytes were further used to grow cartilage-like tissues in rotating shaft bioreactors that demonstrated the potential of baculovirus in cartilage tissue engineering, but their clinical utility in humans is yet to be proved.

Bac-CB-based BMP-2 transduction into human bone marrow-derived mesenchymal stem cells (BMSCs) is also demonstrated to directing ontogenies of naïve BMSCs. Implantation of these transduced cells induced ectopic bone formation in nude mice and promoted calvarial bone repair in immunocompetent rats (Chuang et al. 2009). For massive repairing of bone, sustained expression of genes promoting

osteogenesis (BMP-2) and angiogenesis (VEGF) in adipose-derived stem cells (ASCs) was performed by dual baculovirus vector system. Transplantation of these ASCs in NZW rabbit resulted in accelerated healing, improved bone quality and angiogenesis. Same technique was also tested in rabbits, and the results altogether support the viability of baculoviruses for stem cell engineering and bone formation (Luo et al. 2011).

The propensity of baculoviruses for effective high-level transgene expression has been exploited for cancer gene therapy. Baculovirus vectors have been tailored with suicide, tumour suppressor, pro-apoptotic, immune-potentiating and anti-angiogenesis genes and studied in animal tumour models under in vivo conditions in many anticancer strategies (Luo et al. 2012; Wang and Balasundaram 2010). Recently, stem cells transduced with suicide genes have proved beneficial for curbing primary, solid and metastatic tumours (Zhao et al. 2012).

Today, baculovirus technology has matured to the level that it can be used for plethora of applications. The studies conducted on model organisms in the context of therapeutic applications are encouraging and support further development of baculoviruses from preclinical applications to clinical trials and for human diseases treatment. A deeper and holistic understanding of antigenic and target cell transduction molecular mechanisms will be helpful in enhancing the clinical utility of this unique and powerful gene delivery system.

References

Airenne KJ et al (2013) Baculovirus: an insect-derived vector for diverse gene transfer applications. Mol Ther 21(4):739–749

Ang WX et al (2016) Local immune stimulation by intravesical instillation of baculovirus to enable bladder cancer therapy. Sci Rep 6:27455

Atmar RL et al (2011) Norovirus vaccine against experimental human Norwalk Virus illness. N Engl J Med 365(23):2178–2187

Aucoin MG et al (2007) Virus-like particle and viral vector production using the baculovirus expression vector system/insect cell system. In: Baculovirus and Insect Cell Expression Protocols. Humana, New York, pp 281–296

Bakay RA et al (2007) Analyses of a phase 1 clinical trial of adeno-associated virus-nerve growth factor (CERE-110) gene therapy in Alzheimer's disease: 866. Neurosurgery 61(1):216

Ball JM et al (1999) Recombinant Norwalk virus–like particles given orally to volunteers: phase I study. Gastroenterology 117(1):40–48

Barber GN, Clegg J, Lloyd G (1990) Expression of the Lassa virus nucleocapsid protein in insect cells infected with a recombinant baculovirus: application to diagnostic assays for Lassa virus infection. J Gen Virol 71(1):19–28

Barnett J et al (1990) Human **β** nerve growth factor obtained from a baculovirus expression system has potent in vitro and in vivo neurotrophic activity. Exp Neurol 110(1):11–24

Burke RL (1999) Herpes simplex virus diagnostics. Google Patents

Chen H-C et al (2008) Combination of baculovirus-expressed BMP-2 and rotating-shaft bioreactor culture synergistically enhances cartilage formation. Gene Ther 15(4):309–317

Choi K-S et al (2014) Baculovirus expression of the avian paramyxovirus 2 HN gene for diagnostic applications. J Virol Methods 198:12–17

Chuang C-K et al (2009) Xenotransplantation of human mesenchymal stem cells into immunocompetent rats for calvarial bone repair. Tissue Eng A 16(2):479–488

Chung C-Y et al (2010) Enterovirus 71 virus-like particle vaccine: improved production conditions for enhanced yield. Vaccine 28(43):6951–6957

Cox MM, Hashimoto Y (2011) A fast track influenza virus vaccine produced in insect cells. J Invertebr Pathol 107:S31–S41

Day TP et al (2014) Advances in AAV vector development for gene therapy in the retina. In: Retinal degenerative diseases. Springer, New York, pp 687–693

de Diego ACP et al (2011) Characterization of protection afforded by a bivalent virus-like particle vaccine against bluetongue virus serotypes 1 and 4 in sheep. PLoS One 6(10):e26666

Desrosiers R et al (2009) Use of a one-dose subunit vaccine to prevent losses associated with porcine circovirus type 2. J Swine Health Prod 17(3):148–154

dos Santos CND et al (1992) Trypanosoma cruzi flagellar repetitive antigen expression by recombinant baculovirus: towards an improved diagnostic reagent for Chagas' disease. Biotechnology 10:1474–1477

Dudognon B et al (2014) Production of functional active human growth factors in insects used as living biofactories. J Biotechnol 184:229–239

Eshaghi M et al (2004) Nipah virus glycoprotein: production in baculovirus and application in diagnosis. Virus Res 106(1):71–76

Fauquet CM et al (2005) Virus taxonomy: VIIIth report of the International Committee on Taxonomy of Viruses. Academic, London

Felberbaum RS (2015) The baculovirus expression vector system: A commercial manufacturing platform for viral vaccines and gene therapy vectors. Biotechnol J 10(5):702–714

French T, Marshall J, Roy P (1990) Assembly of double-shelled, viruslike particles of bluetongue virus by the simultaneous expression of four structural proteins. J Virol 64(12):5695–5700

Frey S, Treanor JJ, Atmar RL, Topman D, Chen WH, Ferreira J (2011) Phase 1 dosage escalation, safety and immunogenicity study of a bivalent norovirus VLP vaccine by the intramuscular route. In Annual meeting of the infectious diseases society of America, Boston, 2011

Gaudet D et al (2010) Review of the clinical development of alipogene tiparvovec gene therapy for lipoprotein lipase deficiency. Atheroscler Suppl 11(1):55–60

Gil F et al (2011) Targeting antigens to an invariant epitope of the MHC Class II DR molecule potentiates the immune response to subunit vaccines. Virus Res 155(1):55–60

Goldman B, DeFrancesco L (2009) The cancer vaccine roller coaster. Nat Biotechnol 27(2):129–139

Gómez-Sebastián S et al (2012) Rotavirus A-specific single-domain antibodies produced in baculovirus-infected insect larvae are protective in vivo. BMC Biotechnol 12(1):59

Guyre P et al (2002) Recombinant cat allergen, Fel dI, expressed in baculovirus for diagnosis and treatment of cat allergy. Google Patents

High K et al (2014) Current status of haemophilia gene therapy. Haemophilia 20(s4):43–49

Hu YC, Bentley WE (2001) Effect of MOI ratio on the composition and yield of chimeric infectious bursal disease virus-like particles by baculovirus co-infection: Deterministic predictions and experimental results. Biotechnol Bioeng 75(1):104–119

Janson C et al (2002) Gene therapy of Canavan disease: AAV-2 vector for neurosurgical delivery of aspartoacylase gene (ASPA) to the human brain. Hum Gene Ther 13(11):1391–1412

Jarmin S et al (2014) New developments in the use of gene therapy to treat Duchenne muscular dystrophy. Expert Opin Biol Ther 14(2):209–230

Jiang X et al (1992) Expression, self-assembly, and antigenicity of the Norwalk virus capsid protein. J Virol 66(11):6527–6532

Jiang X et al (1998) Baculovirus-expressed Sapporo-like calicivirus capsid self-assembles into virus-like particles (VLPs) and can be used to detect antibody in children† 854. Pediatr Res 43:148–148

Jiang X et al (2000) Diagnosis of human caliciviruses by use of enzyme immunoassays. J Infect Dis 181(Supplement 2):S349–S359

Kanesashi S-N et al (2003) Simian virus 40 VP1 capsid protein forms polymorphic assemblies in vitro. J Gen Virol 84(7):1899–1905

Kantoff PW et al (2010) Sipuleucel-T immunotherapy for castration-resistant prostate cancer. N Engl J Med 363(5):411–422

Kaplitt MG et al (2007) Safety and tolerability of gene therapy with an adeno-associated virus (AAV) borne GAD gene for Parkinson's disease: an open label, phase I trial. Lancet 369(9579):2097–2105
King AM et al (2011) Virus taxonomy: ninth report of the International Committee on Taxonomy of Viruses. Elsevier, London
Ko Y-J et al (2005) Noninfectious virus-like particle antigen for detection of swine vesicular disease virus antibodies in pigs by enzyme-linked immunosorbent assay. Clin Diagn Lab Immunol 12(8):922–929
Ko Y-J et al (2010) Use of a baculovirus-expressed structural protein for the detection of antibodies to foot-and-mouth disease virus type A by a blocking enzyme-linked immunosorbent assay. Clin Vaccine Immunol 17(1):194–198
Kong XG et al (1997) Application of equine infectious anemia virus core proteins produced in a baculovirus expression system to serological diagnosis. Microbiol Immunol 41(12):975–980
Kost TA, Condreay JP (2002) Innovations—biotechnology: baculovirus vectors as gene transfer vectors for mammalian cells: biosafety considerations. Appl Biosaf 7(3):167–169
Kotin RM et al (1991) Mapping and direct visualization of a region-specific viral DNA integration site on chromosome 19q13-qter. Genomics 10(3):831–834
Krammer F et al (2010) Trichoplusia ni cells (High FiveTM) are highly efficient for the production of influenza A virus-like particles: a comparison of two insect cell lines as production platforms for influenza vaccines. Mol Biotechnol 45(3):226–234
Laurent S et al (1994) Recombinant rabbit hemorrhagic disease virus capsid protein expressed in baculovirus self-assembles into viruslike particles and induces protection. J Virol 68(10):6794–6798
Lee JH et al (2014) Expression of recombinant anti-breast cancer immunotherapeutic monoclonal antibody in baculovirus–insect cell system. Entomol Res 44(5):207–214
Liu L-J et al (2008) Efficient production of type 2 porcine circovirus-like particles by a recombinant baculovirus. Arch Virol 153(12):2291–2295
Liu T et al (2015) Production of a fragment of glycoprotein G of herpes simplex virus type 2 and evaluation of its diagnostic potential. Singap Med J 56(6):346
Long G et al (2006) Functional entry of baculovirus into insect and mammalian cells is dependent on clathrin-mediated endocytosis. J Virol 80(17):8830–8833
López-Macías C et al (2011) Safety and immunogenicity of a virus-like particle pandemic influenza A (H1N1) 2009 vaccine in a blinded, randomized, placebo-controlled trial of adults in Mexico. Vaccine 29(44):7826–7834
Luckow VA, Summers MD (1988) Trends in the development of baculovirus expression vectors. Nat Biotechnol 6(1):47–55
Luo W et al (2011) Baculovirus vectors for antiangiogenesis-based cancer gene therapy. Cancer Gene Ther 18(9):637–645
Luo W et al (2012) Development of the hybrid Sleeping Beauty-baculovirus vector for sustained gene expression and cancer therapy. Gene Ther 19(8):844–851
MacLachlan TK et al (2011) Preclinical safety evaluation of AAV2-sFLT01—a gene therapy for age-related macular degeneration. Mol Ther 19(2):326–334
Maranga L et al (2002) Large scale production and downstream processing of a recombinant porcine parvovirus vaccine. Appl Microbiol Biotechnol 59(1):45–50
Marks WJ et al (2010) Gene delivery of AAV2-neurturin for Parkinson's disease: a double-blind, randomised, controlled trial. Lancet Neurol 9(12):1164–1172
Marx F et al (2001) Diagnostic immunoassays for tick-borne encephalitis virus based on recombinant baculovirus protein expression. J Virol Methods 91(1):75–84
Mathavan S et al (1995) High-level production of human parathyroid hormone in Bombyx mori larvae and BmN cells using recombinant baculovirus. Gene 167(1):33–39
Mauch L et al (1993) Baculovirus-mediated expression of human 65 kDa and 67 kDa glutamic acid decarboxylases in SF9 insect cells and their relevance in diagnosis of insulin-dependent diabetes mellitus. J Biochem 113(6):699–704

Mazur NI et al (2015) Lower respiratory tract infection caused by respiratory syncytial virus: current management and new therapeutics. Lancet Respir Med 3(11):888–900

Mecham J, Wilson W (2004) Antigen capture competitive enzyme-linked immunosorbent assays using baculovirus-expressed antigens for diagnosis of bluetongue virus and epizootic hemorrhagic disease virus. J Clin Microbiol 42(2):518–523

Mena JA et al (2010) Improving adeno-associated vector yield in high density insect cell cultures. J Gene Med 12(2):157–167

Monie A et al (2008) Cervarix™: a vaccine for the prevention of HPV 16, 18-associated cervical cancer. Biol Targets Ther 2(1):107–113

Montross L et al (1991) Nuclear assembly of polyomavirus capsids in insect cells expressing the major capsid protein VP1. J Virol 65(9):4991–4998

Moormann RJ et al (2000) Development of a classical swine fever subunit marker vaccine and companion diagnostic test. Vet Microbiol 73(2):209–219

Mortola E, Roy P (2004) Efficient assembly and release of SARS coronavirus-like particles by a heterologous expression system. FEBS Lett 576(1-2):174–178

Neuzil KM (2016) Progress toward a respiratory syncytial virus vaccine. Clin Vaccine Immunol 23(3):186–188

O'Reilly DR, Miller LK, Luckow VA (1994) Baculovirus expression vectors: a laboratory manual. Oxford University Press on Demand, New York

Osman AA et al (2002) Production of recombinant human tissue transglutaminase using the baculovirus expression system, and its application for serological diagnosis of coeliac disease. Eur J Gastroenterol Hepatol 14(11):1217–1223

Park D-Y et al (2011) Optimization of expression conditions for production of anti-colorectal cancer monoclonal antibody CO17-1A in baculovirus-insect cell system. Hybridoma 30(5):419–426

Pastey MK, Samal SK (1998) Baculovirus expression of the fusion protein gene of bovine respiratory syncytial virus and utility of the recombinant protein in a diagnostic enzyme immunoassay. J Clin Microbiol 36(4):1105–1108

Paton D et al (1991) An ELISA detecting antibody to conserved pestivirus epitopes. J Virol Methods 31(2-3):315–324

Perelygina L et al (2005) Production of herpes B virus recombinant glycoproteins and evaluation of their diagnostic potential. J Clin Microbiol 43(2):620–628

Pham M-Q et al (1999) Human interleukin-2 production in insect (Trichoplusia ni) larvae: effects and partial control of proteolysis. Biotechnol Bioeng 62(2):175–182

Pillay S et al (2009) Optimization of chimeric HIV-1 virus-like particle production in a baculovirus-insect cell expression system. Biotechnol Prog 25(4):1153–1160

Prel A, Le Gall-Recule G, Jestin V (2008) Achievement of avian influenza virus-like particles that could be used as a subunit vaccine against low-pathogenic avian influenza strains in ducks. Avian Pathol 37(5):513–520

Reed JC et al (1992) A strategy for generating monoclonal antibodies against recombinant baculovirus-produced proteins: application to the Bcl-2 oncoprotein. Anal Biochem 205(1):70–76

Rocha-Zavaleta L et al (1997) Differences in serological IgA responses to recombinant baculovirus-derived human papillomavirus E2 protein in the natural history of cervical neoplasia. Br J Cancer 75(8):1144

Roldão A et al (2010) Virus-like particles in vaccine development. Expert Rev Vaccines 9(10):1149–1176

Roy P, Noad R (2008) Virus-like particles as a vaccine delivery system: Myths and facts. Hum Vaccin 4(1):5–12

Saijo M et al (2007) Development of recombinant nucleoprotein-based diagnostic systems for Lassa fever. Clin Vaccine Immunol 14(9):1182–1189

Seppänen H et al (1991) Diagnostic potential of baculovirus-expressed rubella virus envelope proteins. J Clin Microbiol 29(9):1877–1882

Sguazza GH et al (2013) Expression of the hemagglutinin HA1 subunit of the equine influenza virus using a baculovirus expression system. Rev Argent Microbiol 45(4):222–228

Shi X et al (1996) Expression of biologically active human granulocyte-macrophage colony-stimulating factor in the silkworm (Bombyx mori). Biotechnol Appl Biochem 24(3):245–249
Simonelli F et al (2010) Gene therapy for Leber's congenital amaurosis is safe and effective through 1.5 years after vector administration. Mol Ther 18(3):643–650
Smith GE, Summers M, Fraser M (1983) Production of human beta interferon in insect cells infected with a baculovirus expression vector. Mol Cell Biol 3(12):2156–2165
Sugiura T et al (2001) Serological diagnosis of equine influenza using the hemagglutinin protein produced in a baculovirus expression system. J Virol Methods 98(1):1–8
Sumathy S, Palhan VB, Gopinathan KP (1996) Expression of human growth hormone in silkworm larvae through recombinant Bombyx mori nuclear polyhedrosis virus. Protein Expr Purif 7(3):262–268
Summers MD (2006) Milestones leading to the genetic engineering of baculoviruses as expression vector systems and viral pesticides. Adv Virus Res 68:3–73
Sun Y et al (2009) Protection against lethal challenge by Ebola virus-like particles produced in insect cells. Virology 383(1):12–21
van Oers MM, Pijlman GP, Vlak JM (2015) Thirty years of baculovirus–insect cell protein expression: from dark horse to mainstream technology. J Gen Virol 96(1):6–23
Van Rijn P et al (1996) Classical swine fever virus (CSFV) envelope glycoprotein E2 containing one structural antigenic unit protects pigs from lethal CSFV challenge. J Gen Virol 77(11):2737–2745
Virag T, Cecchini S, Kotin RM (2009) Producing recombinant adeno-associated virus in foster cells: overcoming production limitations using a baculovirus–insect cell expression strategy. Hum Gene Ther 28(8):807–817
Wagner R et al (1996) Safety and immunogenicity of recombinant human immunodeficiency virus-like particles in rodents and rhesus macaques. Intervirology 39(1-2):93–103
Wald A, Ashley-Morrow R (2002) Serological testing for herpes simplex virus (HSV)–1 and HSV-2 infection. Clin Infect Dis 35(Supplement 2):S173–S182
Wan C-H et al (1995) Expression of Sendai virus nucleocapsid protein in a baculovirus expression system and application to diagnostic assays for Sendai virus infection. J Clin Microbiol 33(8):2007–2011
Wang S, Balasundaram G (2010) Potential cancer gene therapy by baculoviral transduction. Curr Gene Ther 10(3):214–225
Weyer U, Possee RD (1991) A baculovirus dual expression vector derived from the Autographa californica nuclear polyhedrosis virus polyhedrin and p10 promoters: co-expression of two influenza virus genes in insect cells. J Gen Virol 72(12):2967–2974
Yamaji H (2014) Suitability and perspectives on using recombinant insect cells for the production of virus-like particles. Appl Microbiol Biotechnol 98(5):1963–1970
Yamshchikov GV et al (1995) Assembly of SIV virus-like particles containing envelope proteins using a baculovirus expression system. Virology 214(1):50–58
Yin X et al (2013) Detection of specific antibodies against Tembusu virus in ducks by use of an E protein-based enzyme-linked immunosorbent assay. J Clin Microbiol 51(7):2400–2402
Yoshimatsu K, Arikawa J, Kariwa H (1993) Application of a recombinant baculovirus expressing hantavirus nucleocapsid protein as a diagnostic antigen in IFA test: cross reactivities among 3 serotypes of hantavirus which causes hemorrhagic fever with renal syndrome (HFRS). J Vet Med Sci 55(6):1047–1050
Zhao Y et al (2012) Targeted suicide gene therapy for glioma using human embryonic stem cell-derived neural stem cells genetically modified by baculoviral vectors. Gene Ther 19(2):189–200
Zielonka A et al (2006) Generation of virus-like particles consisting of the major capsid protein VP1 of goose hemorrhagic polyomavirus and their application in serological tests. Virus Res 120(1):128–137
Zincarelli C et al (2008) Analysis of AAV serotypes 1–9 mediated gene expression and tropism in mice after systemic injection. Mol Ther 16(6):1073–1080

Insect RNAi: Integrating a New Tool in the Crop Protection Toolkit

10

Leela Alamalakala, Srinivas Parimi, Navid Patel, and Bharat Char

Abstract

Protecting crops against insect pests is a major focus area in crop protection. Over the past two decades, biotechnological interventions, especially Bt proteins, have been successfully implemented across the world and have had major impacts on reducing chemical pesticide applications. As insects continue to adapt to insecticides, both chemical and protein-based, new methods, molecules, and modes of action are necessary to provide sustainable solutions. RNA interference (RNAi) has emerged as a significant tool to knock down or alter gene expression profiles in a species-specific manner. In the past decade, there has been intense research on RNAi applications in crop protection. This chapter looks at the current state of knowledge in the field and outlines the methodology, delivery methods, and precautions required in designing targets. Assessing the targeting of specific gene expression is also an important part of a successful RNAi strategy. The current literature on the use of RNAi in major orders of insect pests is reviewed, along with a perspective on the regulatory aspects of the approach. Risk assessment of RNAi would focus on molecular characterization, food/feed risk assessment, and environmental risk assessment. As more RNAi-based products come through regulatory systems, either via direct application or plant expression based, the impact of this approach on crop protection will become clearer.

L. Alamalakala (✉) • S. Parimi • N. Patel • B. Char
Maharashtra Hybrid Seeds Company Private Limited,
Dawalwadi, PO Box 76, Jalna 431203, India
e-mail: leela.alamalakala@mahyco.com

D. Kumar, C. Gong (eds.), *Trends in Insect Molecular Biology and Biotechnology*, https://doi.org/10.1007/978-3-319-61343-7_10

10.1 Introduction

Crop protection strategies need continuous improvement and innovation since the ability of the insect herbivores to adapt to any pest control intervention is well documented (Georghiou and Lagunes-Tejeda 1991; Storer et al. 2010). The increased strain on agricultural output due to global challenges such as population growth and climate change in conjunction with the escalating costs of chemical pest control, insecticide resistance, and rising environmental and health concerns creates a need for developing new technologies to close yield gaps and minimize environmental impacts (Pradhan et al. 2015). Sustainable intensification of crop production by using the best of conventional plant breeding (adapted germplasm with native resistance) and the best of biotechnology is a theme that is being widely advocated by the global scientific community to meet the daunting challenge of feeding 9.7 billion in 2050 (Smith 2013). New biotechnological techniques developed during the last two decades have helped agriculture to cope with different challenges like pest resistance, disease, herbicide and stress tolerance, and improved yield and product quality characteristics. Transgenic crops expressing *Bacillus thuringiensis* (Bt) proteins have provided excellent yield protection from insect pest damage, and the success of this technology is evident by the fact that the global planting of crops genetically engineered to express Bt proteins increased to 78 million hectares in 2014 which is a significant fraction of the >170 M ha of transgenic crops cultivated worldwide (James 2014; Baum and Roberts 2014). Although Bt sprays and Bt crops have provided substantial economic and environmental benefits, insect adaptation resulting from the strong selective pressures imposed, has reduced their effectiveness (Tabashnik et al. 2013; Carrière et al. 2015). Therefore, as in the case for synthetic and biological insecticides, alternative modes of action (MOAs) for insect-protected crops are needed, either because some insect species are refractory to Bt proteins or because some have evolved field resistance to the Bt Proteins. To that end, RNA interference (RNAi) which targets and knocks down the expression of genes in a species-specific manner provides significant opportunities for crop protection by managing pest populations and reducing the spread of vector-borne diseases (Price and Gatehouse 2008; Lundgren and Duan 2013). Therefore, there is a growing interest in using dsRNA for insect control, both as a traditional RNAi-based pesticide and RNAi-based genetically modified crop plants.

RNA interference (RNAi) refers to a collection of biological processes, by which exogenously applied and endogenously expressed double-stranded RNAs (dsRNA) target specific endogenous messenger RNAs (mRNAs) for degradation, thereby silencing their expression by making use of conserved cellular machinery (Zamore 2001). Shortly following its discovery in the nematode, *Caenorhabditis elegans* (Fire et al. 1998), RNAi has been observed in a wide range of eukaryotic organisms and has proved itself to be a powerful tool for investigating gene function (Dykxhoorn and Lieberman 2005). In plants, the dsRNA-triggered sequence-specific RNA degradation pathway has been termed post-transcriptional gene silencing (PTGS). The RNAi pathway is a major antiviral system in plants (Szittya and Burgyan 2013) and nematodes (Sarkies and Miska 2013) and serves as a broadly acting (Kemp et al. 2013) and robust antiviral pathway in insects (Nayak et al. 2013; Bronkhorst and

van Rij 2014; Vijayendran et al. 2013). Not only has effective RNAi been demonstrated in many insect species, but it has also been performed in insects in a variety of developmental stages. The evidence for functional RNAi has been reported in a wide range of insect species encompassing different taxonomic groups that include the Coleoptera (Arakane et al. 2004; Suzuki et al. 2008), Diptera (Lum et al. 2003; Dietzl et al. 2007), Dictyoptera, Hemiptera, Hymenoptera (Schluns and Crozier 2007; Antonio et al. 2008), Isoptera, Lepidoptera (Chen et al. 2008; YuQ et al. 2008; Tian et al. 2009; Terenius et al. 2011), Neuroptera, and Orthoptera. Recent studies have shown the potential applications of this tool in fundamental and applied research and more specifically for crop protection against insect pests. The ingestion of double-stranded RNAs targeting essential insect genes by insects can trigger RNAi and lead to growth inhibition, developmental aberrations, reduced fecundity, and mortality. RNAi can therefore be considered as one of a suite of tools for crop improvement and insect protection.

The range of potential applications of RNAi in agriculture is remarkable, and the technology is being evaluated to introduce novel plant traits, increase crop yield, and improve product quality. RNAi is being touted as the "game changer" in agriculture because it has provided a highly specific, non-chemical solution for pest and pathogen control (Baum et al. 2007; Price and Gatehouse 2008; Huvenne and Smagghe 2010). The remarkable systemic nature of this mechanism in insects makes RNAi-based insecticides an exciting new IPM alternative for agricultural pest control (Price and Gatehouse 2008). Proof-of-concept studies clearly illustrate the efficacy of this technology for crop protection. RNAi-based transgenic plants intended for market release can be designed to either induce silencing of target genes *in planta* or in insect pests (Koch and Kogel 2014). RNAi-based GM crops have been developed in the laboratory for three major crops: corn (Baum et al. 2007), cotton (Mao et al. 2007; Mao et al. 2011), and rice (Zha et al. 2011). Although RNAi was perceived to provide greater specificity in pest control and have little or no off-target effects, many studies have shown that unintentional off-target gene silencing in target cells and gene silencing in non-target organisms occur more commonly than expected (Baum et al. 2007; Qiu et al. 2005; Mohr and Perrimon 2012). An overall picture of the field risks, the environmental fate of dsRNA and RNAi effects in variation trials (differential effects of RNAi treatments), is emerging (Chu et al. 2014; Palli 2014; Lundgren and Duan 2013). Our review focuses on the current knowledge of RNAi mechanism in general and highlights the scientific data generated in insects with respect to mechanism, dsRNA uptake, and how RNAi can complement the existing technologies for crop protection for achieving optimum crop productivity.

10.2 RNAi Mechanisms and Machinery

Important insights have been gained in elucidating the detailed mechanism of RNAi, since its initial discovery, and definitions have been proposed to differentiate the various aspects of RNAi in plants and animals. Whangbo and Hunter (2008) categorized the RNAi response into the following three types: cell autonomous, environmental,

and systemic. Environmental and systemic RNAi are together referred to as non-cell autonomous RNAi. Cell autonomous RNAi refers to the silencing effect that is encompassed within the cells where dsRNA is constitutively expressed or exogenously introduced. In the non-cell autonomous RNAi, the silencing signal is directly picked up by cells from the environment, viz., gut or hemocoel. The phenomena in which the silencing signal (siRNA and/or dsRNA) spreads to neighboring cells or remote tissues from an epicenter of cells is called systemic RNAi, whereas in environmental RNAi, RNAi pathway is triggered by environmental exposure (either by soaking or feeding), and this may or may not be followed by systemic movement of the silencing signal (Baum and Roberts 2014). The presence of non-cell autonomous RNAi in arthropods and its specificity are two important factors that paved the way for using this technology in pest control. However, for the successful application of the technology as a crop protection agent, environmental RNAi must first be evaluated, and a suitable delivery system for dsRNA has to be identified.

The RNAi machinery can be categorized into two functional groups: (1) the intracellular machinery, consisting of the Dicer and Argonaute proteins, and (2) the "systemic machinery," composed of factors that amplify the dsRNA trigger and allow it to spread to other tissues within the animal or even to the next generation (Siomi and Siomi 2009; Swevers 2012). The RNAi pathway is initiated upon recognition of the long dsRNA precursor molecule that varies in length and origin and can be introduced into the cell through microinjection, transfection, or expression from endogenous genes (Huvenne and Smagghe 2010; Whangbo and Hunter 2008). dsRNA can also move into the cells through the transmembrane transporters SID1/2 or the endocytosis machinery (Feinberg and Hunter 2003; Jose and Hunter 2007). The dsRNA precursors are processed by Dicer-2, a ribonuclease III (RNase III) family dsRNA endonuclease, into ~ 19–25 nt long siRNA duplexes with characteristic 2 nucleotide (nt) 3′ overhangs. The dsRNA-binding motif proteins (dsRBMs) facilitate the assembly of the siRNAs with the RNase H enzyme Argonaute-2 (Ago2), to form a multi-protein RNA-induced silencing complex (RISC) (Hammond et al. 2001), where one of the siRNA strands (the passenger) is degraded in a process dependent upon Ago2 and the endoribonuclease C3PO (*c*omponent *3 p*romoter *o*f RISC) (Liu et al. 2009). The other strand (the guide) is retained and remains associated with Ago2 and is 2′-0-methylated on its 3′ terminal nt by the Hen1 methyltransferase, thus creating a mature RISC (Horwich et al. 2007; Saito et al. 2007). Base pairing of the guide strand to a complementary target single-stranded RNA (including mRNAs) leads to Ago2-mediated degradation of the target (Meister and Tuschl 2004). The siRNA mechanism of the RNAi pathways is harnessed as an experimental tool to target and degrade specific mRNAs with sequence homology to the administered/incorporated dsRNA molecules.

The fundamental components of the RNAi machinery are evolutionarily conserved among insects and are readily identified in insect species whose genomes have been sequenced (Zhu et al. 2014). The ribonuclease III enzyme Dicer, one of the key enzymes involved in RNAi pathways, is encoded by variable number of genes and presents distinct functions among organisms. While mammals and nematodes have a single Dicer responsible for functions in siRNA and miRNA pathways

(Ghildiyal and Zamore 2009), insects have two Dicer proteins, Dcr-1 and Dcr-2 that are assigned to the miRNA and siRNA pathways (Lee et al. 2004). Dcr-1 preferentially processes the pre-miRNA to miRNA, whereas Dcr-2 is in charge of processing long dsRNA into siRNAs (Tomoyasu et al. 2008; Aronstein et al. 2011; Asgari 2013). miRNAs are processed from endogenous genes and function in the regulation of gene expression, while the siRNAs are derived from dsRNA molecules and provide defense against invading viruses. The argonaute family proteins (AGO) are the central protein components of the silencing complexes (RISC) that act in mediating target recognition and silencing (Peters and Meister 2007) and have been observed in different insect taxonomic groups (Aronstein et al. 2011; Swevers et al. 2013). The Ago proteins with a proven role in determining RNAi efficiency were found to be duplicated in the *Tribolium castaneum* genome (Tc-Ago-2a and Tc-Ago-2b), whereas *Drosophila* carries only one copy of the AGO-2 gene, thereby suggesting a relationship between number of copies of AGO gene and insect RNAi response (Tomoyasu et al. 2008).

In animals, the uptake of dsRNA is facilitated by two machineries, viz., the transmembrane channel-mediated uptake machinery based on SID-1(systemic interference defective-1) and SID-2 proteins (Jose and Hunter 2007) and the endocytosis-mediated uptake machinery (Saleh et al. 2006). In *C. elegans*, the SID-1 protein is inferred to function as a dsRNA channel (Winston et al. 2002), and the SID-2 has been implicated in dsRNA uptake by gut cells and probably functions in environmental RNAi (Jose and Hunter 2007). SID-1 homologues are detected in the genomes of insects belonging to Coleoptera, Lepidoptera, Hymenoptera, and Hemiptera but not Diptera (Gordon and Waterhouse 2007). Although putative insect orthologs of the *C. elegans sid* genes have been described in various insect species, their involvement in RNAi is still not known (Xu and Han 2008; Huvenne and Smagghe 2010). The *sid-1*-like genes of insects show greater sequence homology with the *C. elegans* gene *chup-1*, a cholesterol transporter that has no involvement in RNAi, than to *sid-1* (Valdes et al. 2012; Luo et al. 2012). In *Drosophila melanogaster*, which lack a SID gene ortholog, dsRNA uptake occurs by receptor-mediated endocytosis (Saleh et al. 2006). In the nematode *C. elegans* and in many plants, there exists a host-derived RNA-dependent RNA polymerase (RdRp), for amplifying the silencing signals by generating “secondary siRNAs” that sustain the RNAi response (Carthew and Sontheimer 2009). There is no evidence of such RdRp homologue in any insect genome sequenced to date (Tomoyasu et al. 2008). There is considerable ambiguity on how the RNAi triggered by the acquisition of dsRNA molecules is sustained in insect cells, and it is speculated that some unknown mechanism may be responsible for the systemic RNAi response (Barnard et al. 2012).

10.3 Important Considerations of an RNAi Experiment

Although RNAi is a highly conserved cellular mechanism and its use for the control of insect herbivores seems to be very straightforward, RNAi application and efficacy remain variable between genes, life stages, and organisms. Factors that

determine the success of RNAi experiments in different insect species include the uptake of dsRNA (environmental RNAi and/or systemic RNAi), presence/absence of the core RNAi machinery, cellular uptake and propagation of signal (Roignant et al. 2003; Miller et al. 2008), and dsRNA degrading enzymes (Arimatsu et al. 2007), as well as other differences in genetic backgrounds (Kitzmann et al. 2013; Scott et al. 2013). These biological variables have been experimentally studied in different insect species. A detailed description of different factors influencing the success of RNAi technology in insects is presented below.

10.3.1 Identification of Target Genes

For successful RNAi, the choice of the essential genes that can trigger a lethal RNAi response in the insect pest requires careful consideration. Target gene selection is crucial yet challenging especially for those pest species that are usually difficult to rear in the lab or those that lack the required genomic and genetic tools for a whole animal-high-throughput-screen. In such cases, data from appropriate insect model systems can be subjected to large scale unbiased RNAi screens for target gene identification (Ulrich et al. 2015). The abundance of target gene transcript and the rate of protein turnover are two important factors that influence the outcome of an RNAi experiment. An mRNA pool with high turnover that codes for a protein with a short half-life is considered an ideal gene target for RNAi (Scott et al. 2013). Phenotypic evaluation of gene function using RNAi may not be easy for a stable protein with a long half-life. Another limitation is that for majority of genes, mRNA turnover and protein half-life are not known. Potential target genes which will function under field conditions can be identified by performing bioassays that closely mimic the conditions in the field. A precise choice of the target region from the target gene for dsRNA synthesis will ensure the specificity of RNAi and concurrently limits the off-target effects. Identification of essential targets is possible by extended literature search, analyses of available DNA/RNA sequence databases, and gene screening mediated by second-generation sequencing (Andrade and Hunter 2016; Wang et al. 2011). Insect genomics research initiatives have steadily increased in the last two decades due to the availability of cost-effective, high-throughput DNA sequencing platforms that have contributed to the sequencing of genomes of agriculturally important organisms. These efforts have broad implications in insect functional genomics studies, enhancing the throughput of RNAi target identification and development of insect management technologies.

10.3.2 Designing the RNAi Molecule

When designing RNAi experiments, important considerations regarding the design of a specific RNAi molecule (in the form of dsRNA, siRNA, or a hairpin RNA) for a target gene of interest (GOI) include the length of the molecule, sequence identity to the target transcript of the insect, and the region targeted within the mRNA

(Scott et al. 2013; Andrade and Hunter 2016). The length of dsRNA is an important parameter for successful RNAi as different efficacies by different sizes of dsRNA were reported by Whyard et al. (2009) and Saleh et al. (2006). Although the minimal required length to achieve an optimum RNAi effect varies among insect species (Bolognesi et al. 2012), greater success with insect RNAi has been achieved with dsRNA molecules of ≥50–200 bp in length (Huvenne and Smagghe 2010). Huvenne and Smagghe (2010) provide a comprehensive survey of the length range of dsRNAs used in early studies: from 134 to 1842 bp, with most studies using 300–520 bp. The advantages of using longer >200 bp dsRNA for RNAi strategies in pest management are the production of many siRNAs against the targeted mRNA transcript, potentially maximizing the RNAi response. Occasionally, designing of RNAi molecules, shorter in length than ideal, may achieve desired specificity. Studies have shown the effectiveness of chemically synthesized siRNAs (obtained by dicing the dsRNA in vitro before delivery to the insect) in the suppression of target gene expression.

Another important aspect in the design of dsRNA sequences is the stringency to be adopted in order to achieve the desired specificity and avoid/minimize off-target effects. This property facilitates the designing of species-specific sequences that mediate insect lethality and is a prerequisite for taking the technology from the laboratory to the field. As reported by Zhang et al. (2010), two genes with high sequence similarities can both be silenced by the same dsRNA. This has important implications in off-target effects. Therefore the target gene and target region should be carefully determined in order for adequate and specific RNAi. The gene regions (e.g., 5′ or 3′ end of the gene) to which RNAi molecules are designed have also yielded variable results, thus emphasizing the importance of screening multiple RNAi sequences for a gene of interest (Mao and Zeng 2012; Pridgeon et al. 2008; Loy et al. 2012).

The RNAi molecule design process can be aided by software tools, algorithms, and databases that evaluate the genome sequence and RNA folding kinetics to optimize effectiveness. Following are some of the online software tools that are available to minimize off-target effects and achieve specific and precise silencing effect: the NEXT-RNAi software enables the design and evaluation of siRNAs and long ds-RNAs and can be used for the design and evaluation of genome-wide RNAi libraries in an organism-independent manner for all sequenced and annotated genomes. The input data for the analysis are the desired target sequences and an off-target database. The Next-RNAi software was deployed to design novel genome-wide RNAi libraries of long dsRNA for the following insects, viz., *D. melanogaster*, *T. castaneum*, and *Anopheles gambiae*, and to design multiple RNAi for a specific gene to study associated phenotype (Horn et al. 2010). The web-based E-RNAi tool initially developed for RNAi experiments in *C. elegans* and *Drosophila* (Zeynep et al. 2005) provides siRNA and long dsRNA design suggestions suitable for RNAi experiments in a variety of other species and insects that include *Apis mellifera*, *T. castaneum*, *Acyrthosiphon pisum*, *A. gambiae*, and *Aedes aegypti* (Horn and Boutros 2010). It can calculate off-target impacts that may affect the phenotypic results. The dsRNA sequences are evaluated for their specificity and efficiency. The dicer

enzyme in the RNAi machinery cleaves long dsRNA into small 19–22 nucleotides long siRNAs. dsCheck is a software that investigates individual 19 nucleotide fragments of long dsRNA and produces a list of potential off-target gene candidates based on its novel algorithm. This tool provides off-target search to verify previously designed dsRNA sequences and also presents "off-target minimized" dsRNA design (Naito et al. 2005).

10.3.3 Delivery of dsRNA for Insect RNAi

The effective introduction of the RNAi trigger into an organism and its subsequent entry into the RNAi pathway is the most limiting factor of the RNAi experiment. There are many methods of dsRNA delivery reported and applied in RNAi experiments, including microinjection (Tan et al. 2008; Martin et al. 2006), feeding (in vitro synthesized dsRNA) (Zhou et al. 2008; Zhu et al. 2011), transgenic plants expressing dsRNA(Baum et al. 2007; Mao et al. 2007), nanoparticle RNAi (Zhang et al. 2010), soaking (Terenius et al. 2011; Ulvila et al. 2006), and topical application (Pridgeon et al. 2008). Intracellular RNAi results from the expression of hairpin RNAs as transgenes or during the introduction of dsRNA into cells by electroporation or transfection or by direct delivery into a cell. Extracellular RNA, which requires the uptake of dsRNA molecules by the cells, is achieved by soaking, feeding, or injection into the hemocoel (Yu et al. 2013). From the studies it is evident that most insect RNAi studies relied on the delivery of specific dsRNA triggers through either microinjections (Fire et al. 1998; Adams et al. 2000) or ingestion through feeding (Ulvila et al. 2006; Rangasamy and Siegfried 2012). Each of these methods has its own advantages and limitations which are discussed further.

10.3.3.1 Delivery of dsRNA Trigger Through Injection (Microinjection)

Microinjection is a widely used dsRNA delivery method in arthropods, and the first successful microinjection experiment performed in vivo was in *D. melanogaster* embryos in which the expression of *frizzled* and *frizzled* 2 genes was down regulated by intracellular RNAi (Kennerdell and Carthew 1998). This technique was subsequently used to deliver dsRNA to the giant silk moth, *Hyalophora cecropia* (Bettencourt et al. 2002). Although, the sequencing of *D. melanogaster* genome in 2000 (Adams et al. 2000) made RNAi a popular research tool in functional genomics of this model insect, extracellular RNAi seems to have limited application in this species (Dzitoyeva et al. 2001). A plausible reason for this could be the fact that the cells in most larval tissues seem to be recalcitrant to the uptake of dsRNA from outside the cell (Miller et al. 2008), whereas some tissues in the adults are able to take up dsRNA (Tomoyasu et al. 2008), thus limiting the use of RNAi in gene function studies. In *T. castaneum*, a stored product pest, and a coleopteran model insect, microinjection of dsRNA, both in larvae and adults, is widely used in functional genomics studies, as this species shows a robust systemic RNAi response (Tomoyasu and Denell 2004). Comprehensive microinjection protocols for RNAi experiments

have been published for the two model insects, *Tribolium* and *Drosophila*, and these protocols provide a quick reference and standard for similar experiments in other arthropod species. Successful delivery of dsRNA by injection has also been demonstrated in Lepidoptera; however, the method has shown great variation in effectiveness between species and is not as simple as shown in other taxa (Terenius et al. 2011; Kolliopoulou and Swevers 2014). Nonetheless, quite a number of RNAi microinjection experiments have been performed in species from the order Lepidoptera with most notable success achieved with *Bombyx mori* and *Manduca sexta*, and the members of the Saturniidae family were found to be quite sensitive to RNAi using hemocoel injection as the dsRNA delivery method, compared to other species within the order (Yu et al. 2013). In the moth species, RNAi based on dsRNA microinjection has been applied to all life stages, viz., egg (Osanai-Futahashi et al. 2016; Fabrick et al. 2004), larvae (Mohammed et al. 2015; Sun et al. 2016; Zhao et al. 2013), pupa (Choi et al. 2012; Qian et al. 2015), and adults (Abrieux et al. 2013; Hassanien et al. 2014). Injection of dsRNA has been proven successful to cause a knockdown effect in the economically important model insect, the *A. mellifera* (Farooqui et al. 2003; Gatehouse et al. 2004; Aronstein and Saldivar 2005).

Microinjection has also been used to deliver dsRNA or siRNA for RNAi in the agriculturally important hemipteran herbivores, the pea aphid, *A. pisum* (Jaubert-Possamai et al. 2007; Mutti et al. 2006), whitefly, *Bemisia tabaci* (Ghanim et al. 2007), and nymphs and adults of the small brown plant hopper, *Laodelphax striatellus* (Liu et al. 2010). Microinjection of long dsRNA into the body cavity of *B. tabaci* caused downregulation of genes uniquely expressed in the midgut and salivary glands, and injection of dsRNA targeting the whitefly *Drosophila* chickadee homologue caused phenotypic effects in the ovaries of *B. tabaci*. The disruption of gene expression in the hemipteran herbivores opens the door to new strategies aimed at curbing down the deleterious effects of these insect pests to agriculture (Ghanim et al. 2007).

Microinjection protocols are currently available for various taxa, including Lepidoptera, Diptera, Hymenoptera, the Orthopterans, and Cockroaches (Terenius et al. 2011; Blandin et al. 2002; Martin et al. 2006; Belles 2010; Huang and Lee 2011; Nakamura et al. 2008). Microinjection has been applied to all life stages in hemi- and holometabolous insects, and a large variation in the success rates of these experiments has been observed between different species, genera, and taxa (Yu et al. 2013). In the case of larvae, injections are usually carried out dorsally or between segments, whereas in adults the tissue under the wings is the easiest location to inject the organism. Microinjection has both its advantages and disadvantages compared to the other methods of dsRNA delivery. This technique allows researchers to get the dsRNA directly and effectively into the tissue of choice or into the hemolymph without being hindered by barriers such as the integument or the gut epithelium, in addition, to providing the flexibility to deliver the precise amount of dsRNA. However, an important shortcoming of this technique in insects is the mechanical damage during the injection, which is quite significant when targeting embryos and neonatal larvae and pupae (Scott et al. 2013; Yu et al. 2013). The mechanical damage may also have undesirable effects or even obscure the targeted

effects especially when studying the function of genes relation to behavior and survival using RNAi. Furthermore, this method is time-consuming and labor-intensive, requires expertise, can only be used in the laboratory, and is not suitable for RNAi-based pest control (Xu et al. 2016).

10.3.3.2 Delivery of dsRNA Trigger Through Ingestion

Feeding is another method for introducing dsRNA into an organism for triggering RNAi. RNAi triggered by ingested dsRNA was first demonstrated in *C. elegans* (Timmon and Fire 1998; Timmons et al. 2001) and subsequently applied in various insects and taxa such as *Spodoptera exigua*, *Diabrotica virgifera virgifera*, and *Epiphyas postvittana* (Turner et al. 2006; Baum et al. 2007; Tian et al. 2009; Surakasi et al. 2011). The dsRNA used for ingestion experiments can either be expressed in bacteria or plants, or they can be synthesized in vitro and then fed to insects either by mixing with food or by supplying as solution droplets. Uptake of bacterially expressed dsRNA was applied in *S. exigua* to suppress the expression of *S. exigua* chitin synthase A (SeCHSA) gene, a non-midgut gene specifically expressed in the cuticle and trachea of *S. exigua* (Tian et al. 2009). The study further established that the phenotypes recovered post-ingestion were dependent on the dsRNA dosage and accumulation. Transcriptional suppression of target gene expression in salivary glands of the tick, *Ixodes scapularis* (Soares et al. 2005), and fat body tissue of *Reticulitermes flavipes* (Ulvila et al. 2006) was observed when dsRNA was delivered through ingestion route. The oral delivery of in vitro synthesized dsRNA either by dissolving the dsRNA in liquid artificial diets (Sadeghi et al. 2009) or overlaying on the surface of solid foods was used in *T. castaneum*, *A. pisum*, and *M. sexta* for the knockdown of a species-specific E-subunit of the vATPase gene (Whyard et al. 2009), which led to 50–75% mortality in all three insect species (Yu et al. 2013). RNAi triggered by ingested dsRNA that was delivered via artificial diet surface coated with dsRNA or food that was mixed with dsRNA was effective in *Drosophila* species (Whyard et al. 2009). Transient suppression of β1 integrin subunit (βSe1) expression in *S. exigua* gut epithelium was achieved by providing dsRNA-treated cabbage leaf disks to fourth instar larvae, and significant mortality was also recorded (Surakasi et al. 2011).

The dsRNA droplet feeding as described by Turner et al. (2006) is yet another method applied in the research on a larval gut carboxylesterase gene (*Epos*CXE1) and the adult antennae-expressed pheromone binding protein (EposPBP1) gene in *E. postvittana* larvae and a cytochrome P450 (CYP6BG1) gene in *Plutella xylostella* (Bautista et al. 2009). Successful knockdown of the target gene and significant RNAi effects were observed in these studies proving the efficacy of ingestion as a method for effective RNAi. Nanoparticle-mediated RNAi technique, in which dsRNAs were entrapped by the polymer chitosan via electrostatic forces to form a chitosan/dsRNA nanoparticle, was another innovation for delivering the dsRNA to insect by ingestion (Zhang et al. 2010). Formation of nanoparticles was believed to enhance the efficacy of RNAi by providing improved stability to the dsRNA molecule through the delivery process (Yu et al. 2013). Oral delivery of dsRNA can also be achieved by exposing the target insects to transgenic plants that express hairpin

dsRNAs targeting specific genes from insects to increase their resistance to herbivorous insects (Baum et al. 2007; Mao et al. 2007). Silencing of genes in target insects of Lepidoptera, Coleoptera, and Hemiptera was evaluated effectively by delivery of dsRNAs through transgenic plants (Baum et al. 2007; Pitino et al. 2011; Zha et al. 2011). Model plants such as thale cress (*Arabidopsis thaliana*) (Zha et al. 2011; Liu et al. 2015), tobacco (*Nicotiana tabacum*) (Mao et al. 2007), rice (*Oryza sativa*) (Zha et al. 2011), tomato (*Solanum lycopersicum*) (Mamta and Rajam 2016), and cotton (*Gossypium hirsutum*) (Mao et al. 2011) were transformed to express dsRNA against target herbivores.

Oral delivery of dsRNA into insects for RNAi can thus be performed by any of the following approaches, viz., artificial diet, detached plant parts, or intact plants, and provides several advantages. Not only is this technique easy to perform, it is a labor-saving, cost-effective, and comparatively less invasive method with the potential for high-throughput screening of target genes and potential for field application (Tian et al. 2009; Kamath et al. 2000). This method may be the most suitable method for developing RNAi pesticides since it allows RNAi through pest insect feeding on sprayed dsRNA-based pesticide or transgenic plant and bacteria that express dsRNA (Xu et al. 2016). The limitations of oral delivery of dsRNA include the limited or no efficiency of dsRNA ingestion in inducing RNAi in some insect species, thereby suggesting that the technique may not be suitable for all species. In *S. litura*, ingested dsRNA targeting a gut-specific aminopeptidase N failed to induce RNAi (Rajagopal et al. 2002). The gut environment of the target insect species and the final effective dosage/concentration delivered or needed for RNAi are difficult to determine and optimize, which could compromise the investigations (Turner et al. 2006; Surakasi et al. 2011).

10.3.3.3 Delivery of dsRNA Trigger Through Soaking and Transfection

Soaking, as a method for successful delivery of dsRNA and induction of specific RNAi response, was first reported in *C. elegans* (Tabara et al. 1998) and subsequently applied to large-scale analysis of gene function in nematodes and other species. This method is particularly suitable for RNAi analysis in insect cell lines and tissues, as well as in specific life stages of insects, such as eggs and neonate larvae (Yu et al. 2013; Wang et al. 2011; Wu et al. 2016; Singh et al. 2013). The S2 cells derived from *D. melanogaster* embryos were used for initial soaking experiments by adding specific dsRNA to the cell growth medium to suppress specific gene expression, and then subsequently this method became the most commonly used method for inducing RNAi response in S2 cells (Clemens et al. 2000; Caplen et al. 2000; Shah and Förstemann 2008). Since then, RNAi experiments in *Sf21* cells derived from ovaries of *S. frugiperda* were conducted using this technique, in which the downregulation of target genes was accomplished by soaking the cells in dsRNA (Sivakumar et al. 2007) and siRNA (Agrawal et al. 2004) solutions. It was however observed that simple soaking of the cells in dsRNA supplemented culture medium was not sufficient to trigger an RNAi response in some species, but introduction of the dsRNA into the cells by transfection was probably more efficient technique

when compared to soaking (Beck and Strand 2005; Valdes et al. 2003). Soaking appears to work with similar efficiency as feeding in *C. elegans*; however, it is not as effective as microinjection (Tabara et al. 1998). The method is easy to use and is suitable for conducting high-throughput RNAi screens (Perrimon and Mathey-Prevot 2007) and genome-wide analysis in the study of phenotypes characterization (Sugimoto 2004).

10.3.3.4 Other dsRNA Delivery Methods

Other methods to introduce dsRNA trigger into organisms, including electroporation (Osanai-Futahashi et al. 2016), virus-mediated delivery (Kontogiannatos et al. 2013), and bacterial symbiont mediated delivery (Whitten et al. 2016), have also been applied to RNAi in insects including moths (Xu et al. 2016).

10.3.4 Impact of dsRNA Dosage on RNAi

Optimal concentration of dsRNA uptake by an insect determines the final outcome of RNAi, and the dose required varies with insect species, developmental stage of the insect, the abundance of target gene transcript, and the delivery method used. A higher dose is usually required when the RNAi molecule is delivered orally as compared to injection. For RNAi studies using microinjection, it is not possible to use very high concentrations because of the viscous nature of the dsRNA solutions. Although very high doses of dsRNA can be incorporated into the insect artificial diets, synthesizing large amounts of dsRNA may not be cost-effective, thereby presenting a major experimental bottleneck (Scott et al. 2013). The presence of nucleases in various extracellular fluids of insects, such as the saliva of *Lygus lineolaris* (Allen and Walker 2012), the digestive juices of *B. mori* (Arimatsu et al. 2007), and the hemolymph of *M. sexta* (Garbutt et al. 2013), also impacts the dosage of RNAi trigger that can cause effective RNAi as the breakdown of dsRNA may not provoke an optimum RNAi response. In the pea aphid, *A. pisum*, a lack of response in RNAi feeding and injection assays was associated with the degradation of dsRNA by both the salivary secretions and the hemolymph (Christiaens et al. 2014). Finally, insect gut pH is yet another factor that influences the efficiency of RNAi. It has been observed that gut pH is quite variable among insect orders with the pH being predominant acidic in Coleopteran larvae to strongly alkaline in some species of Lepidoptera. All of these biochemical factors in the gut greatly influence the stability of dsRNA and impact the bioavailability of dsRNA to suppress target gene expression (Price and Gatehouse 2008). The strong alkaline gut pH in the Lepidopteran larvae may be a contributing factor in the species from this order for being recalcitrant to gene silencing by RNAi. The effects of RNAi in insects usually begin to appear within 4 to 5 days post-ingestion suggesting that there may be a dose response (Yu et al. 2013). In several insects, the RNAi efficiency is either low or variable at best, and therefore a relatively high dosage of RNAi trigger molecules is needed to compensate for the species- and tissue-specific antagonistic biological factors, viz., degradation of dsRNA and weak activity of the RNAi machinery that

impact the efficacy of RNAi. Furthermore, the mode of uptake, the ability to process RNAi molecules, and the ability to spread the signal are other important factors that influence the essential dose required to induce an RNAi response.

10.3.5 Evaluation of RNAi Experiments

The desired result of an RNAi experiment varies with the objective of the study (gene function analysis or insect control). For research attempting to develop novel RNAi-based product for insect control, a successful outcome would be to obtain high insect mortality, whereas for gene function analysis, physiological indices of predicted function should be central to the analysis. Therefore, defining and integrating the appropriate physiological and fitness assays in the experimental design are critical (Scott et al. 2013). Furthermore, in order to expedite the process of development of a viable pest control product using RNAi strategy, identifying the best delivery mechanism (i.e., topical sprays, baits, or transgenic plants) early in the product development phase is of utmost importance (Andrade and Hunter 2016).

10.3.5.1 Bioassays

RNAi bioassays for insects are optimized taking into consideration the feeding behaviors of insects, and the in vitro experiments are designed to mimic conditions the insects will encounter in the field. A range of concentrations of dsRNA are tested to select an optimal concentration for effective RNAi. For insects with piercing-sucking mouthparts, artificial feeding bioassays are being used widely for RNAi. However, major drawbacks associated with liquid feeding bioassays (dsRNAs mixed in a liquid diet or a sucrose solution) include the high mortality levels observed in the controls, the significantly higher dsRNA concentrations required to achieve mortality, and the increased degradation of dsRNA in the liquid diet due to bacterial or fungal contaminations (Upadhyay et al. 2011). Also, it has been reported that concentrations of up to 1 μg/μL cannot be reproduced inside plant vascular tissues (Borgio 2010; Katoch et al. 2013; Tomizawa and Noda 2013), which is an important parameter to consider when developing an effective RNAi control strategy against the plant sap feeders (Andrade and Hunter 2016). Successful oral uptake of dsRNA delivered via host plants treated with dsRNA either as a foliar spray or root drench was demonstrated in two hemipteran insects, the xylem-feeding leafhopper (*Homalodisca vitripennis*) and the phloem-feeding Asian citrus psyllid (*Diaphorina citri*) (Hunter et al. 2012). Cost-effective feeding bioassays for screening large number of dsRNA molecules against the hemipteran pests can be developed using leaf disks, whole leaf, new growth leaves and stem, or root cuttings (Andrade and Hunter 2016). These bioassays can be terminated after 8–10 days of observations for mortality and may be extended further to record observations on insect oviposition, egg viability, or nymph development, since the plant material was found to remain viable for up to 40 days on an average. These bioassays provide the flexibility to screen for the synergistic effects of multiple dsRNAs in addition to screening single dsRNA against multiple insect herbivores of a specific host plant (Andrade and Hunter 2016).

For bioassays in chewing insects, viz., the Lepidoptera and Coleoptera, which are foliage feeders, topical foliar spray is a suitable method for delivery of dsRNA. Host plant leaves sprayed with dsRNA solution are fed to the insect, and the RNAi effects are evaluated. The effectiveness of this procedure was reported with the Coleopteran insects such as western corn rootworm (WCR) (Bolognesi et al. 2012), Colorado potato beetle (CPB) (Miguel and Scott 2016), the diaprepes root weevil (DRW), and *Diaprepes abbreviatus* L. (Andrade and Hunter 2016) and in several other species as well. In WCR, the dsRNA delivered as a foliar spray silenced genes in tissues far from the gut epithelium, and in CPB, the actin dsRNA conferred protection against insect damage for at least 28 days under greenhouse conditions, and the dsRNA was found to be quite stable. Since chewing insects tend to consume a lot of leaf material each day, a low-dose spray may be able to deliver a significant amount of RNAi trigger.

10.3.5.2 Controls

When conducting RNAi experiments, depending on the type of assay/treatment, including a negative control, viz., empty vector, empty cassette, buffer only, or a non-specific control (such as dsGFP (green fluorescent protein) gene region), is essential as this will aid in discriminating specific gene silencing from the simple induction of siRNA processing machinery by exposure to a dsRNA. The reporter dsRNA used as a negative control is selected on the basis of having no off-target effects; hence, it should not show sequence similarity to any known insect mRNA transcript.

10.3.5.3 Quantifying the Transcript Levels

The most commonly used method for tracking effectiveness of RNAi is the RT-qPCR, a method that measures the successful reduction of transcript levels as a result of RNAi and expressed as a percent reduction of the relevant transcript in the treatment group versus the negative control group. Although this method is widely used and accepted, the choice of the reference or housekeeping genes for calculating relative transcript levels is challenging, and these genes although identified and validated on the species level show variable expression depending on the physiology of the insect and the tissue being targeted.

The final phenotypes obtained as a result of a successful RNAi experiment depend on the reduction of protein levels for the gene of interest; therefore, it is highly desirable to determine relative protein concentration. However, there is a possibility of no correlation between the protein levels and the level of transcript suppression; in addition a distinct phenotype may not be observed or recovered despite successful suppression of transcript levels especially when redundancy is built into a specific biological function (Scott et al. 2013). Proteins with a long half-life interfere with the phenotypic changes. For example, a significant phenotypic change in spinosad sensitivity was not observed when RNAi was used to suppress the expression of an $\alpha 6$ nicotinic acetylcholine receptor subunit involved in spinosad toxicity in both *D. melanogaster* and *T. castaneum*, suggesting that the RNAi may not be an appropriate method to study the role of target genes where the protein is stable for longer periods (Rinkevich and Scott 2013).

10.4 Insecticidal RNAi and Crop Protection

The potential of insecticidal RNAi for crop protection and management of insect herbivores and beneficial insects is widely recognized, and the following options, viz., transgenic plants expressing the insecticidal RNAi trait (plant-mediated RNAi) and using dsRNA as a traditionally applied insecticide, are being pursued by industry and academia for product development (Xue et al. 2012; Lundgren and Duan 2013). RNAi offers exquisite specificity and flexibility that cannot be matched by other crop protection interventions such as chemical insecticides, biological control, or protein-coding transgenes (Scott et al. 2013). The breakthrough in applying insecticidal RNAi strategy for controlling agricultural pests via transgenic plants expressing hairpin dsRNA to target specific gene regions of the insect pests came from studies conducted on the western corn rootworm, *D. v. virgifera* (WCR) (Baum et al. 2007), and cotton bollworm, *Helicoverpa armigera* (CBW) (Mao et al. 2007). Baum et al. (2007) screened 290 gene targets for evaluating RNAi response in larval WCR, from which they observed rootworm mortality or stunting in approximately 2/5 of the targets screened at concentrations as low as ~50 ng/cm^2 in surface overlay diet bioassays (Baum et al. 2007; Baum et al. 2011). A distinct phenotypic response was not observed on suppression of certain gene targets (Baum and Roberts 2014). A gossypol-induced cytochrome P450 gene, *CYP6AE14*, was targeted for RNAi analysis in CBW by Mao et al. (2007). The gene is expressed in the larval midgut and permits the bollworm to tolerate inhibitory concentration of the cotton secondary metabolite, gossypol. When CBW larvae were fed *A. thaliana* or *N. tabacum* leaves expressing *CYP6AE14* dsRNA, lower expression levels of this transcript was observed in the midgut, and larval growth was retarded, and both effects were more dramatic in the presence of gossypol. The demonstration of plant resistance to insects mediated by the RNAi-based trait has not only added a new tool to the crop protection toolkit but has exemplified the following key issues for successful environmental RNAi in crop pests: a large number of specific gene targets are available that can be screened, the choice of the target sequence(s), the size of the RNAi trigger, and the mode of delivery of the RNAi trigger (Baum et al. 2007; Mao et al. 2007; Bolognesi et al. 2012; Khajuria et al. 2013).

Due to the long product development timelines, slow regulatory approval of plant delivered RNAi, and the recalcitrant nature of species in some taxa toward environmental RNAi, the future of the RNAi-based insect pest management strategies may depend on non-transformative RNAi strategies and development of topical formulations (Hunter et al. 2010; Baum and Roberts 2014). Alternative approaches are being developed for using RNAi strategy as a conventional topically applied pesticide. The use of topical sprays relies on the penetration or adsorption of the RNAi trigger through the insect cuticle, thereby bypassing the insect gut (Wang et al. 2011). For achieving commercial success with the RNAi strategy as a conventional topically applied pesticide, efficient methods for production, delivery, and increased stability of dsRNA have to be developed. Nanoparticle-mediated RNAi was found to be an effective delivery method and provided a better stability of dsRNA in some insects (Yu et al. 2013; Zhang et al. 2010; Palli 2014). The delivery of dsRNA using

nanoparticles reduces dsRNA degradation and increases cellular uptake of intact dsRNA (Joga et al. 2016). The perceived advantages in using chitosan nanoparticles such as being low-cost, enabling high-throughput evaluation of phenotypes (Mysore et al. 2014), and being nontoxic besides their biodegradable nature (Dass and Choong 2008) make them a novel tool for dsRNA delivery (Zhang et al. 2010). Chitosan nanoparticles were used to demonstrate gene knockdown effects in *A. gambiae* (Zhang et al. 2015) and diet-based delivery of chitosan nanoparticles suppressed gene expression in Asian corn borer (He et al. 2013). Recently, topical application of pathogen-specific dsRNA for virus resistance in plants was reported (Mitter et al. 2017). dsRNA was loaded on designer, nontoxic degradable, layered double hydroxide (LDH) clay nanosheets, and the complex is referred to as "BioClay." BioClay offered protection against cucumber mosaic virus (CMV) and pepper mild mottle virus (PMMov) in the local lesion or systemic infection assays. LDH-based nanoparticle technology can also be used in similar way to offer insect protection in plants.

Cost-efficient methods for the production of vast amounts of dsRNA are being optimized and include bacterial, plant, and synthetic production (Palli 2014; Andrade and Hunter 2016). The use of bacteria to synthesize and deliver dsRNA is being pursued for managing agricultural pests especially in non-major crops, such as vegetables and fruits, and for developing insecticidal baits for urban pests, such as ants, cockroaches, and termites (Zhou et al. 2008; Ratzka et al. 2013). The delivery of dsRNA through bacteria and viruses and improving the RNAi efficiency through use of nanoparticles, liposomes, and/or chemical modifications are discussed in Joga et al. (2016). Many of the main crop pest species have already been targeted by RNAi technology using various genes and delivery methods. The first RNAi-based product as a spray is expected for market release during 2017/18 (Joga et al. 2016).

10.4.1 Coleoptera

RNAi is quite effective in insects that belong to order Coleoptera (Tomoyasu et al. 2008), and this fact has been corroborated through studies conducted in different species from the order. The utility of RNAi in both basic and applied science has been demonstrated in the beetle species, and they appear to be the first target group to be controlled by the new generation of RNAi transgenics (Palli 2012; Palli 2014; Rodrigues and Figueira 2016). Small quantities of ingested dsRNA appears to be sufficient to initiate RNAi response in beetles, such as the western corn rootworm, Colorado potato beetle, southern corn rootworm, *Diabrotica undecimpunctata* howardi, and the canola flea beetle, *Phyllotreta striolata* (Baum et al. 2007; Bolognesi et al. 2012; Zhao et al. 2008; Baum and Roberts 2014), which was evident by low LC_{50} values (1–10 ppb). This level of sensitivity to ingested dsRNA was not observed in insect species outside the order Coleoptera, and the sensitivity was exhibited by both the larval and adult stages (Rangasamy and Siegfried 2012; Zhao et al. 2008), although the in vivo amplification of dsRNA/siRNA has not been shown in beetles. While systemic RNAi is functional in most beetle species studied, variable RNAi

responses have been documented across the species, notably to orally delivered dsRNA. RNAi does not seem to work uniformly in all beetles, as illustrated in studies with red flour beetle, *T. castaneum*, and cotton boll weevil, *Anthonomus grandis* (Baum et al. 2007; Whyard et al. 2009). The western corn rootworm is one of the most important agricultural pests in which plant-mediated RNAi was successfully demonstrated. Significant larval stunting and mortality were observed in the WCR feeding on maize roots that express a hairpin version of the housekeeping gene vacuolar ATPase (vATPase). The maize roots showed less injury as well (Baum et al. 2007). Comprehensive studies of RNAi in corn rootworm by Baum et al. (2007) provided important insights into the parameters for successful RNAi, for example, their study showed that screening a large number of gene targets through simple surface overlay diet bioassays at relatively low concentration of ~50 ng/cm^2 was effective in identifying suitable targets causing lethal phenotypes for successful environmental RNAi in corn rootworm. At least 2/5 of the total 290 gene targets screened were found to cause rootworm mortality or stunting (Baum et al. 2007; Baum et al. 2011). Specifically, the study by Baum et al. (2007) reported no significant difference in efficacy of six ~300 bp dsRNAs corresponding to the V-ATPase region in target gene knockdown in WCR suggesting that a single dsRNA of this size is optimum for RNAi in rootworms. Growth inhibition and mortality were observed in adults fed with vATPase dsRNA-treated artificial diet containing the feeding stimulant cucurbitacin as bait (Rangasamy and Siegfried 2012). In this study, mRNA levels were found to decrease within 24 h of ingestion of dsRNA; however, decrease in protein levels was observed only after 3 days of feeding. The RNAi effect resulted in mortality, although complete suppression of protein was not achieved. A detailed study of the corn rootworm Snf7 ortholog (DvSnf7), which encodes an essential protein involved in intracellular trafficking showed that dsRNAs of greater than or equal to approximately 60 base pairs (bp) are required for the initiation of biological activity in artificial diet bioassays (Bolognesi et al. 2012). Additionally, 21bps short interfering (si) RNAs are not taken up by the midgut cells and therefore failed to trigger the silencing of Snf7 gene, supporting the size versus activity relationship observed in diet bioassays.

Parental RNAi (pRNAi) is an RNA interference response where the gene knockdown phenotype is observed in the progeny of the treated organism (Vélez et al. 2017). In this type of RNAi, the uptake of dsRNA that targets genes regulating embryonic development by adults results in reduced egg hatch rates or complete absence of viable larvae, with the adults remaining unaffected (Khajuria et al. 2015; Fishilevich et al. 2016). A recent study by Vélez et al. (2017) probed the parameters for successful parental RNAi in WCR for two target genes the chromatin remodeling gene *brahma* (brm) and the gap gene *hunchback* (*hb*). The parameters investigated included the concentration, duration, and timing of exposure, with respect to the mating status in WCR females, and the effects of *brm* and *hb* dsRNA on male sperm viability and fecundity were also evaluated. Results from this study demonstrate that all parameters studied affect the strength of pRNAi phenotype in females and very subtle effects on sperm count were observed in males. These diet-based pRNAi studies thus provide a framework for developing the technology for field

level testing of plant-based pRNAi. Hu et al. (2016) reported the discovery of two new gene targets, the *dvssj1* and *dvssj2*, in WCR that are orthologs of Drosophila genes snakeskin (*ssk*) and *mesh*, respectively. Oral delivery of dsRNA targeting *dvssj1* and *dvssj2* through diet-based insect feeding assays demonstrated target gene suppression, larval growth inhibition, and mortality. Transgenic plants expressing dsRNA of *dvssj1* were protected from WCR damage and showed insecticidal activity.

Since the leading insect model organism, *D. melanogaster*, lacks a robust systemic RNAi response, Tomoyasu et al. (2008) analyzed the genes involved in RNA-mediated gene silencing and the systemic RNAi response in *T. castaneum*. These studies showed that *T. castaneum* contains a relatively larger inventory of core component genes than *D. melanogaster* that probably is responsible for the observed sensitivity of this coleopteran species to dsRNA. Functional analysis of three *Tribolium* homologues of *C. elegans* sid-1 genes suggested that *T. castaneum* sid-like genes are not required for systemic RNAi. Target genes having clear RNAi phenotypes in the model insect *T. castaneum* were studied further in *D. v. virgifera* larvae, to test the efficacy of RNAi for target-site screening. Delivery of dsRNA of *D. v. virgifera* orthologs of laccase 2 (*DvvLac2*) and *chitin synthase 2* (*DvvCHS2*) by injection resulted in prevention of post-molt cuticular tanning and reduced chitin levels in midguts, respectively, thus providing a tool for identifying potential insecticidal target in western corn rootworm (Alves et al. 2010).

Colorado potato beetle (CPB), a notorious insect pest on solanaceous vegetables potatoes, tomatoes, and eggplants, has not only developed resistance against insecticides but also has an exceptional ability to detoxify plant chemicals. Transgenic potato plants expressing dsRNA targeting CPB genes demonstrated limited success. Feeding dsRNA expressed in bacteria was found to work very well in killing CPB (Palli 2014). In insects species where long dsRNA is more effective than siRNA for effective environmental RNAi, plant-mediated RNAi may not be effective against insect herbivory if there are low levels of dsRNA in the tissue due to the presence of the endogenous plant RNAi pathways that processes dsRNAs into short interfering RNAs. This bottleneck was addressed by expressing the dsRNA in chloroplasts (which lack an RNAi machinery), thereby improving the levels of dsRNA needed to provide protection (Zhang et al. 2015). Transplastomic potato plants expressing hairpin versions of B-actin and *Shrub* genes in the chloroplasts conferred complete plant protection from insect herbivory; a dramatic increase in the dsRNA levels that induced up to 100% mortality of the CPB in only 5 days was also observed in these plants.

10.4.2 Lepidoptera

The order Lepidoptera (moths, butterflies, and skippers) represents not only the second largest order in the class Insecta, but also includes major pests of agricultural importance (Xu et al. 2016). The lepidopteran insect herbivores were successfully managed by the first-generation insecticidal plants expressing the Bt proteins for

nearly two decades; however, recent reports of resistance evolution to Bt proteins have created the need to find alternatives to manage these pests. Consequently, the lepidopterans were one of the first and main targets for RNAi crops. However, studies have highlighted that in Lepidoptera RNAi has many times proven to be difficult to achieve, and a large variation in response was observed across species (Terenius et al. 2011; Xu et al. 2016). Furthermore, a review of the experimental data of RNAi in Lepidoptera also revealed interesting trends, viz., RNAi is particularly effective in the family Saturniidae, genes involved in immunity are good RNAi targets, knockdown of gene expression in the epidermal tissue seems to be the most difficult to achieve, and high dsRNA dosages are needed for silencing genes by oral delivery of dsRNA (Terenius et al. 2011). Comparative studies of RNAi response in the Coleopteran (robust RNAi response) and Lepidopteran insects (poor RNAi response) undertaken to understand the varying RNAi efficiency in these two insect orders suggest that despite efficient uptake of dsRNA by the Lepidopteran and Coleopteran cell lines, the dsRNA was degraded faster in the Lepidoptera. Furthermore, experimental evidence showed that the dsRNA was processed to siRNA in Coleoptera but not in Lepidoptera thus suggesting that dsRNA degradation, poor intracellular transport of dsRNA, reduced accessibility of dsRNA to the RNAi machinery, and reduced activity of the RNAi machinery are the likely factors for poor RNAi response in Lepidopteran insects (Shukla et al. 2016).

The first lepidopteran RNAi experiments were reported in 2002, where the knockdown of a pigment gene following the dsRNA injection into *B. mori* embryos (Quan et al. 2002), the silencing of hemolin gene expression by heritable RNAi in *H. cecropia* embryos (Bettencourt et al. 2002), and a study on a putative *Bacillus thuringiensis* toxin receptor in *Spodoptera litura* (Rajagopal et al. 2002) were reported. An increasing number of reports were published since 2007, and particularly after 2010, that describe the successful RNAi experiments in moth species using classic and novel dsRNA delivery methods, viz., microinjection, feeding, soaking, electroporation, and transgenic insect technique, as well as viral-mediated, bacterial-mediated, and plant-mediated dsRNA uptake. A comprehensive review of the recent progresses of the RNAi technique in moths has been published by Xu et al. (2016). Chemically synthesized dsRNA or siRNA was used to demonstrate and validate gene function in initial studies in Lepidoptera, and in later studies it was used to identify suitable targets for RNAi-based crop protection. In all of these studies, the dsRNA was delivered to the insects either by incorporation of the dsRNA into artificial diet, by droplet feeding, or by treating the leaf tissue prior to feeding. The first example of gene silencing via oral delivery of dsRNA was reported by Turner et al. (2006) in the brown apple moth, *Epiphyas postvittana* (Walker), to achieve suppression of several target genes by droplet feeding of 4000 ppm dsRNA solution. Subsequently, silencing via oral uptake of dsRNA was reported in a wide range of lepidopteran species, but the objective of these studies was not to illustrate the RNAi-mediated insect control or mortality but to investigate gene function in a metabolic or developmental process by selectively suppressing the target gene expression (Belles 2010). Some examples of these early RNAi studies mediated by oral delivery of dsRNA include the down-regulation of a cytochrome P450, CYP6BG1 in the diamondback moth,

Plutella xylostella, which reduced the larval resistance to permethrin (Bautista et al. 2009); the knockdown (63–64% reduction of transcript level) of a gut-specific chitinase gene (OnCht) in the European corn borer (ECB), *Ostrinia nubilalis* larvae, which facilitated an understanding of the regulation of chitin content in the peritrophic matrix (PM) of ECB (Khajuria et al. 2010); the suppression of β1 subunit integrin (βSe1) gene expression from the beet armyworm, *S. exigua*, to study its role in cellular immune response and larval development (Surakasi et al. 2011); and a 5 μL drop of 3μg SfT6 dsRNA was used in feeding assays to demonstrate the role of a serine protease gene in the processing of the *B. thuringiensis* Cry1Ca1 insecticidal protein in the fall armyworm (Rodriguez et al. 2010). High concentrations of dsRNA (50–2500 ppm) were used in all cases, and a concentration-dependent mortality was reported upon silencing of the βSe1 subunit gene in the beet armyworm (Surakasi et al. 2011) and the vacuolar ATPase E subunit gene in *M. sexta* (Whyard et al. 2009). A vacuolar ATPase-A gene and an arginine kinase gene were targeted in the tomato leafminer, *Tuta absoluta*, an invasive lepidopteran insect pest that is a major threat to commercial tomato production worldwide causing yield losses of up to 100% in various regions (Desneux et al. 2011; Camargo Barbosa et al. 2016). The uptake of dsRNA by the moth larvae from tomato leaflets treated with in vitro synthesized dsRNA resulted in approximately 60% reduction in transcript accumulation in the larvae for both the targets selected, increased larval mortality and protection against insect herbivory (Camargo Barbosa et al. 2016).

Several studies in Lepidoptera also used chemically synthesized siRNA to suppress target gene expression because of the difficulties faced in delivering sufficient dsRNA to the Lepidopteran gut epithelial cells (Gong et al. 2011). Feeding siRNAs specific to acetylcholine esterase AChE to *H. armigera* larvae at ~0.35 ppm along with the artificial diet resulted in a 15% increase in insect mortality followed by other phenotypes which include growth inhibition of larvae, pupal weight reduction, malformation, and lower fecundity as compared to the control larvae (Kumar et al. 2009). Similar results were reported when acetylcholine esterase genes AChE1 and AChE2 genes were targeted in *P. xylostella* using chemically synthesized and modified siRNAs (Gong et al. 2013). The siRNAs were modified by addition of a dTdT overhang in the 3′end, 2′-methyl-nucleotides, and 5′ polyethylene glycol (PEG), and this sodium salt formulation contained chitosan. In this study it was found that silencing of *PxAChE2* caused higher mortality compared to *PxAChE1*, thus confirming the importance of *PxAChE2* in *P. xylostella*. In the laboratory cabbage leaf bioassays, one siRNA, Si-ace2_001, at a concentration of 3 μg cm^{-2} displayed the best insecticidal activity causing 89.0% mortality and exhibited LC_{50} and LC_{90} values of 53.7 μg/mL and 759.71 μg/mL, respectively (Gong et al. 2013). In the field evaluation, *P. xylostella* larvae feeding on *Brassica oleracea* and *B. alboglabra* treated with different siRNA doses had no negative effects on plant morphology, color, and growth of vein; however, Si-ace2_001 in the dose of 200 ppm was moderately harmful to the larvae with a mortality of 58.8% 5 days after exposure (Gong et al. 2013). These studies suggest that siRNA can be readily taken up by insect larvae with their diet, and there might not be a strict dsRNA size dependency to the environmental RNAi in lepidopterans (Baum and Roberts 2014).

The uptake of large dsRNA expressed in *Escherichia coli* has also been reported to impact the growth and survival of the lepidopteran larvae. In *S. exigua*, silencing a *chitin synthase A* by feeding the larvae with bacterial culture expressing dsRNA caused larval mortality to increase by 14%, 21%, 26%, and 18% in the first-instar larvae, fourth and fifth larval instars, the prepupae, and pupae, respectively (Tian et al. 2009). Silencing the *CYP6B6* gene by feeding larvae with bacteria expressing dsRNA caused a 27% increase in larval mortality in *H. armigera* (Zhang et al. 2013). Targeting the expression of arginine kinase (AK), an important regulation factor of energy metabolism in invertebrates, by delivering the dsRNA to larvae through diet containing bacteria expressing *arginine kinase* dsRNA caused larval mortality in *H. armigera* to increase by 2–11% (Qi et al. 2015). A major limitation in these studies was that neither the concentration of dsRNA in diet nor the effect of dsRNA alone was reported (Baum and Roberts 2014). Yang and Han (2014) reported the evaluation of different dsRNA delivery methods in *H. armigera* and concluded that continuous ingestion of the bacteria expressing dsRNA was detrimental to insect development and survival than naked dsRNA and the naked dsRNA degraded much faster in the midgut than in hemolymph. Feeding-based RNAi mediated by dsRNA expressed in bacteria or synthesized in vitro, of a molt-regulating transcription factor *CiHR3* in Sugarcane stem borer, *Chilo infuscatellus* Snellen, caused significant abnormalities and weight loss in insects within 7 days of treatment (Zhang et al. 2012). However, silencing a juvenile hormone esterase-related gene via bacterial delivery of dsRNA did not result in a phenotype in the corn stalk borer, *Sesamia nonagrioides* (Kontogiannatos et al. 2013).

Transgenic plants expressing insect-specific dsRNAs have been considered as a promising strategy for improving pest resistance to insect herbivory in crops, and therefore studies were undertaken in Lepidoptera to demonstrate the suppression of gene expression via plant-mediated dsRNA delivery. Most studies reported so far that used transgenic plants have targeted genes in *H. armigera* to suppress the development and survival of the moth pest (Xu et al. 2016). Significant suppression of the ecdysone receptor (EcR) gene expression was observed in *H. armigera* larvae that fed on tobacco plants expressing EcR dsRNA, and it resulted in significantly higher lethality (40%) compared to the gfp control group (10%). Moreover, the growth of the larvae fed on leaves of transgenic tobacco plants expressing *HaEcR* dsRNA was significantly delayed, their body sizes reduced, and the larvae died with significant molting defects (Zhu et al. 2012). Elevated mortality and developmental aberrations were reported in the larvae of the beet armyworm when fed on the same transgenic tobacco tissue, probably because of the shared sequence similarity of the EcR target sequences in these two species (Zhu et al. 2012).

Xiong et al. (2013) reported larval mortality of 22–30% and >50% mass reduction in *H. armigera* that fed on transgenic tobacco leaf disks expressing the dsRNA of a molt-regulating transcription factor (HaHR3). Transgenic cotton plants expressing a dsRNA derived from the *H. armigera* gossypol-inducible cytochrome P450 *CYP6AE14* did not cause mortality in *H. armigera* larvae that fed on the transgenic tissue; however the plants showed increased tolerance to insect herbivory (Mao et al. 2011). An increase in larval stunting was achieved by co-delivering a cysteine

proteinase to damage the larval peritrophic matrix that led to higher gossypol accumulation (Mao et al. 2013). This study suggests that plant damage by insect herbivory can be mitigated by targeting detoxification mechanism in the insect midgut, since it appears to not require a systemic RNAi response in the insect (Baum and Roberts 2014). A recent study used transgenic tobacco (*Nicotiana tabacum* var. Xanthi) and tomato (*Solanum lycopersicum* Mill cv. Pusa early dwarf) plants expressing *H. armigera* chitinase dsRNA (Mamta and Rajam 2016) to show that RNAi-induced mortality in *H. armigera* larvae that fed on transgenic tissue increased by up to 45%. Transient expression of the dsRNA of the vacuolar ATPase-A gene and an arginine kinase gene of *T. absoluta*, in the tomato plants by infiltration of *Agrobacterium* cells carrying binary plasmids expressing the target gene hairpin constructs, and uptake of dsRNA by the larvae by feeding on this tissue conferred plant protection against insect feeding damage and reduced target transcript accumulation in the larvae and associated lethality. This study provides evidence that RNAi could be a promising alternative approach for the control of *T. absoluta* (Camargo Barbosa et al. 2016).

The delivery of dsRNA targeting larval stage-specific transcripts, as a topical application at 50 ppm, was found to cause significant gene silencing and larval mortality at 5 days post-spray in the Asian corn borer, *Ostrinia furnacalis* (Wang et al. 2011). From this study it was inferred that sprayed dsRNA could have either directly penetrated the body wall and reached the target site via the hemolymph or reached the site of action via the tracheoles to produce RNAi effect. It was also reported that significant RNAi-induced lethality was observed in many of the treatments despite the absence of significant gene silencing at day 3; this raises pertinent questions about the role of a non-RNAi mechanism in the effects observed or the sensitivity of the method used for measuring transcript knockdown (Wang et al. 2011).

10.4.3 Hemiptera

Hemipterans (whiteflies, aphids, leafhoppers, and plant hoppers) representing major agricultural pests of crops have piercing sucking mouthparts that are inserted into the plant vascular system. These pests inflict direct damage to plants by sucking sap and indirect damage as vectors transmitting plant pathogens particularly plant viruses (Price and Gatehouse 2008). The systemic insecticides predominantly used to control sap-sucking insects pests have contributed to insecticide resistance and high residual activity. Since these pests are recalcitrant to Bt proteins, novel control strategies have to be developed to manage them. The development of an RNAi-based trait provides a good option for controlling hemipteran herbivores; however, the outcome of this strategy relies on the effective delivery of the dsRNA through the vascular tissues (Andrade and Hunter 2016).

RNAi experiments have been successfully confirmed in the hemipteran herbivores encompassing several economically important pests such as the whitefly (*Bemisia tabaci*), the brown plant hopper (*Nilaparvata lugens*), the pea aphid (*A. pisum*), and plant bugs. In hemipterans, as with other insect orders, early studies

were aimed at studying gene function and not insect mortality or pest control (Belles 2010; Paim et al. 2013). These experiments revealed that the oral delivery of dsRNA for gene silencing is an attractive alternative to microinjection in Hemiptera because of the relatively small size and fragile nature of the immature stages (Baum and Roberts 2014). Furthermore, for using RNAi trait in crop protection, oral uptake is a preferred route for dsRNA delivery to insect body, although microinjection was the typical mode of delivery in many of the successful experiments. Difficulties have also been reported in achieving optimum RNAi in some Hemiptera, including *A. pisum* and the tarnished plant bug, *Lygus lineolaris* (Allen and Walker 2012), due to the limited persistency of the RNAi trigger in the insect body. Across all RNAi experimental data available for Hemiptera to date, it has been observed that there is substantial amount of variability in the dietary concentrations of dsRNA required for knocking down gene expression levels and/or obtaining lethal phenotypic effects, and response ranging from very low to complete knockdown of the transcripts was reported (Baum and Roberts 2014; Christiaens and Smagghe 2014). This variation is seen not only between different species within the order but even between experiments conducted within the same organism. It has been observed that the Hemiptera require a much higher dietary concentrations of dsRNA, viz., at least three orders of magnitude higher than the effective concentrations used with the coleopteran species (Baum and Roberts 2014). These studies also provide valuable insights on the best approaches and targets for using RNAi as a pest control strategy.

Gene silencing following ingestion of dsRNA delivered via artificial diets and transgenic plants has been reported for several hemipteran insects. The v-ATPase subunit E gene knockdown in *A. pisum* and associated mortality (Whyard et al. 2009), suppression of gene expression by 41–48% in *N. lugens* (Li et al. 2011), the silencing of *aquaporin 1* (ApAQP1) gene leading to elevated hemolymph osmotic pressure in A. pisum (Shakesby et al. 2009) and in the same species, depletion in the expression of gap gene *hunchback* (Ap*hb*), a key regulator in the antero-posterior patterning causing the expression of a lethal phenotype (Mao and Zeng 2012), silencing of the gene trehalose phosphate synthase (NlTPS) gene in *N. lugens* causing suppression of transcript expression, disturbed development and lethality in the planthoppers (Chen et al. 2010), and the downregulation of the Ecdysone receptor (SaEcR) and ultraspiracle protein (SaUSP) genes of the grain aphid *Sitobion avenae* F. that impacted aphid survival and fecundity (Yan et al. 2016) are some examples of gene silencing and/or associated phenotypes following ingestion of dsRNA in Hemiptera. Upadhyay et al. (2011) reported silencing of the ribosomal protein L9 (RPL 9) and vacuolar ATPase subunit A in the whitefly, *Bemisia tabaci*, upon ingestion of dsRNA/siRNA, with the LC_{50} values of 11.21 and 3.08 µg/mL, respectively. More than 80% mortality was observed when the target gene expression was knocked down in whiteflies, and the insects showed remarkably higher sensitivity to siRNA. Wuriyanghan et al. (2011) demonstrated the induction of specific RNAi effects in the potato/tomato psyllid (*B. cockerelli*) by using a modified artificial feeding system containing 15% sucrose, food coloring, and Cy™3-labeled dsRNA. Their study reported that significant RNAi effects were observed when

dsRNAs were provided at high concentrations (500 ng/μL or 1000 ng/μL), and this observation was consistent with those reported in other insects such as light-brown apple moth (*E. postvittana*) and pea aphid (*A. pisum*) (Turner et al. 2006; Shakesby et al. 2009). In grain aphid, *S. avenae*, feeding large dsRNA for multiple gene targets (selected after transcriptome profiling) at dietary concentration of 7.5 ng/μL resulted in downregulation of target gene expression, mortality, and developmental stunting of the aphids (Zhang et al. 2013). This study reports achieving lethal RNAi phenotypes at relatively low concentrations of dsRNA (7.5 ppm), which will minimize the risks associated with off-target effects of using high dsRNA dosages and thus facilitate the application of plant-mediated RNAi for developing insect-proof plants. Furthermore, a homologue of Coo2 (a protein effector that promotes host plant colonization in *A. pisum* (Mutti et al. 2008)) from grain aphid *SaCoo2*, was found to cause increased mortality in the *S. avenae* RNAi experiments; however, how the knockdown of this gene impacts aphid feeding behavior on artificial diets is ambiguous (Mutti et al. 2008; Zhang et al. 2013). Feeding high concentration of *Inhibitor of apoptosis* (*IAP*) dsRNA (1000 ppm) failed to elicit any detrimental effect in *L. lineolaris* nymphs (Allen and Walker 2012), although silencing of the same target gene via dsRNA injection resulted in mortality (Walker and Allen 2011). In the corn plant hopper, *Peregrinus maidis*, two genes encoding subunits of *P. maidis* V-ATPase (*V-ATPase B* and *V-ATPase D*) were chosen as RNAi target genes, and two delivery methods, viz., oral delivery (500 ng/uL) and microinjection, were evaluated to investigate the effectiveness of RNAi (Yao et al. 2013). Both methods of dsRNA delivery resulted in knockdown of target transcripts; however, with microinjection a reduction of 27-fold in the normalized abundance of V-ATPase transcript 2 days post-injection was observed as compared to a two-fold reduction after 6 days of oral ingestion. The injection method was more rapid and effective, and although prolonged suppression of (day 6) the VATPase D transcript resulted in a detectable lethal phenotype, it was observed at a time point where significant mortality was observed in the control insects as well (Yao et al. 2013; Baum and Roberts 2014). This experiment highlights the limitation of the artificial diet experiments for RNAi studies in Hemiptera, where the phenotypes caused by environmental RNAi are slow to manifest and keeping the insects alive on an artificial diet for >7 days can be a major challenge. Consequently, plant-mediated RNAi, viz., the *in planta* production of dsRNA of essential insect genes, has been a preferred method for the proof-of-concept studies in Hemiptera.

The plant-mediated RNAi effects of three genes expressed in the midgut of *N. lugens* were studied (Zha et al. 2011), and although target gene expression was suppressed in the hoppers feeding on the GM rice plants, no lethal phenotype was detected. RNAi activity was demonstrated by targeting *Myzus persicae* genes expressed in gut (*Rack*1, a receptor of activated kinase) and salivary glands (*MpCoo2*) (Pitino et al. 2011). Gene expression was knocked down by up to 60% when aphids were fed on *N. benthamiana* leaf disks transiently producing dsRNA corresponding to these genes and on *A. thaliana* plants stably producing the dsRNAs. A decrease in the fecundity of *M. persicae* was observed that was consistent with these genes having essential functions; however, no lethal effects were

observed. Injection of Coo2 siRNA into pea aphid adults (*A. pisum*) resulted in a dramatic depletion of the target salivary gland transcript, and the aphids injected with siCoo2-RNA died well before the control aphids injected with green fluorescent protein (Mutti et al. 2006), suggesting the greater efficiency of microinjection over plant-mediated dsRNA uptake for controlling this species. A similar outcome, viz., reduced fecundity but no mortality, was observed when *M. persicae* fed on *A. thaliana* expressing dsRNA of a serine protease gene (Bhatia et al. 2012) and tobacco plants expressing dsRNA targeting the *hunchback* (hb) gene (Mao and Zeng 2014).

Three target genes (*Rak1*, *MpCoo2*, and *MpPIntO2*) with different functions in aphids were selected to study the persistence and trans-generational effects of plant-mediated RNAi in the green peach aphid (Coleman et al. 2015). This study demonstrated that for the three genes examined RNAi-mediated downregulation and persistence levels in the aphids were not influenced either by the gene sequence or the function; however, a continuous supply of dsRNA was required to maintain the RNAi effect since insects lack genes encoding an RNA-dependent RNA polymerase (RdRP), the enzyme necessary for the siRNA amplification step that leads to persistent RNAi effects (Sijen et al. 2001). The finding that the RNAi effect is transferred to the next generation in aphids revealed by the downregulation of target genes in nymphs born from mothers exposed to dsRNA-producing transgenic plants renders plant-mediated RNAi as a powerful tool for aphid control (Coleman et al. 2015). More recently, it was shown that transgenic tobacco lines expressing long dsRNA precursors of *v-ATPaseA* provided resistance to whiteflies by delivering sufficient siRNA to knockdown the whitefly *v-ATPase* gene expression. A significant silencing response leading to whitefly mortality was recorded in whiteflies feeding on transgenic plants (Thakur et al. 2014). A comprehensive review of the plant-mediated RNAi studies reveals that the dsRNA produced by the plants is processed into short siRNA molecules by the plants own RNAi machinery. The presence of long dsRNA in the plant phloem was mentioned in only one report. These data suggest that the sap-sucking insects are mainly taking up siRNA rather than longer dsRNA, although it has been suggested that the RNAi machinery in insects mainly responds to dsRNA (Christiaens and Smagghe 2014).

10.5 RNAi Risk Assessment and Regulation

As RNAi-based technologies for crop improvement, pest control, and therapeutic applications are developed and products utilizing these technologies are gearing up for market release, assessment of perceived risks will be of prime importance. The potential risks associated with the RNAi-based technologies can be categorized and assessed under molecular characterization, food/feed risk assessment, and environmental risk assessment. Molecular characterization focuses on establishing effects due to off-target gene silencing that may occur due to sufficient sequence homology between small RNAs and mRNAs influencing the function or process (Lundgren and Duan 2013; Ramon et al. 2014). Identifying these off-target genes would

facilitate to understand associated risks. Off-target gene silencing may occur both in the RNAi-based GM plant and also in the organisms feeding on the plant. These organisms include target pests and nontarget organisms (NTOs). It should be noted that not all off-target silencing events would lead to significant reduction in gene expression and/or result in detectable phenotypic changes (Casacuberta et al. 2014). Non-availability of genome data of plants/varieties being used for introduction of RNAi-based transformation event and genome data of nontarget organisms limits the use of bioinformatics-based approaches for NTO risk assessment.

The food/feed risk assessment process follows comparative approach to identify intended and unintended changes that may occur in a GM plant. The comparative assessment includes proximate analysis, analysis of, compositional characteristics, toxicity, allergenicity, and nutritional characteristics of the GM plant. This strategy for evaluating potential food/feed risks of RNAi-based GM plants is accepted to be appropriate by various regulatory agencies worldwide (FSANZ 2013; US EPA 2014). The choice of which component and characteristics to be chosen for compositional and agronomic evaluation is determined during the hazard identification step of problem formulation process and is partly based on their ability to predict harm. It should be noted that unintended effects are by nature not expected naturally and so are difficult to test for them directly (Ladics et al. 2015; Schnell et al. 2015; Devos et al. 2015).

The environmental and ecological risk assessment has been discussed by various authors (Auer and Frederick 2009; Lundgren and Duan 2013; Ramon et al. 2014; Vélez et al. 2017; Roberts et al. 2015). This primarily includes the adverse effects on nontarget organisms, environmental fate, and risk of resistance evolution in target pests. The potential adverse effects on nontarget organisms can be studied following tier-based approach (US EPA 2007; Rose 2007; EFSA 2010a, b; ILSI-CERA 2011; Romeis et al. 2011, 2013). The tier-based assessment involves controlled laboratory studies in lower tiers to field-based studies in highest tier. The early-tier studies assess the adverse effects due to direct exposure to the insecticidal protein at concentrations that are several folds higher than the environmental exposure concentrations (US EPA 2007; Raybould 2011; Romeis et al. 2013). The potential for adverse ecological effects of MON 87411 maize, which expresses DvSnf7 RNA, was studied and reported by Bachman et al. (2016). An assessment plan with the routes and levels of exposure and testing representative functional taxa was developed, and the potential for toxicity of DvSnf7 RNA was evaluated. The test nontarget organisms (NTOs) included predators, parasitoids, pollinators, and soil biota besides aquatic and terrestrial vertebrate species. Endpoint observations recorded included survival, growth, development, and reproduction, and results of their study demonstrated no adverse effects with any species tested at, or above, the maximum expected environmental concentration (MEEC). All margins of exposure for NTOs were >tenfold the MEEC. They concluded that exposure to DvSnf7 RNA, both directly and indirectly, is safe for NTOs at the expected field exposure levels.

Surrogate species or model species are used to conduct early-tier studies (Romeis et al. 2008). In general the surrogate species are selected based on exposure pathway, knowledge on activity and mode of action, amenability of test system, and

availability of test organism. However, when risk assessment of RNAi products are being considered, the surrogate species should be selected based on phylogenetic relationship to the target organism, as the surrogate would likely be susceptible due to sequence homology/similarity (Romeis et al. 2013). This necessitates evaluating additional or different surrogate species from those tested for *Bt* crops (Vélez et al. 2017). Additionally, the susceptibility or unresponsiveness of a model organism to the dsRNA in environment may help in selecting correct surrogate species for NTO studies (Roberts et al. 2015). Among arthropod orders, coleopterans are more sensitive to dsRNA (Belles 2010), and lepidopterans require high concentrations of dsRNA to elicit a response as compared to coleopterans (Ivashuta et al. 2015). The observed differences in RNAi efficiency in insects make it even more difficult and complex in choosing surrogate or model organisms in the risk assessment process.

In most of the NTO studies, the measurable endpoint has been mortality, and limited information exists on effects other than mortality (Vélez et al. 2017). Considering that the effects of RNAi are not completely understood, endpoints other than mortality need to be considered through standardized methods (Auer and Frederick 2009; Vélez et al. 2017). Recently, the risks of RNAi-based GE crops on a nontarget soil micro-arthropod, *Sinella curviseta*, a decomposer, were tested through RNAi dietary toxicity assay, and the endpoint measurements included gene expression profiles, survival, and life history traits (Pan et al. 2016). *S. curviseta* larvae developed significantly faster under the treatments of dsDVV and dsSC than the vehicle control, and results of this study suggest that the impacts of ingested arthropod-active dsRNAs on this representative soil decomposer are negligible. The selection and use of reference genes for RT-qPCR analysis in *Coccinella septempunctata* to assess unintended effects of RNAi GM plants was studied and reported by Yang et al. (2016). This study will be a critical step toward the development of an in vivo dietary RNAi toxicity assay for assessing the risks associated with RNAi transgenic plants.

Higher-tier studies in semi-field, greenhouse, or open field conditions are undertaken only when an adverse effect is detected in lower-tier studies. Long-term field assessment of effects of Bt cotton and Bt corn showed minor or negligible risks to nontarget species in these ecosystems (Daly and Buntin 2005; Head et al. 2005; Lawo et al. 2009; Li and Romeis 2010; Naranjo 2005; Torres and Ruberson 2005, 2007). In a recent study reported by Ahmad et al. (2016), potential impact of GM corn MON 87411 (expresses insecticidal dsRNA transcript and Cry3Bb1 protein besides CP4EPSPS protein) on nontarget arthropods (NTAs) was evaluated in the field. They evaluated NTA abundance and damage among GM corn and comparators. Twenty taxa met minimum abundance criteria, out of which nine were considered to be representative of corn ecosystems. They conclude that there is no adverse environmental impact of MON87411 on NTAs compared to conventional corn and demonstrate utility of relevant transportable data for risk assessment in other corn regions. The higher-tier studies may not always find adverse impacts on NTOs due to the complexity of ecosystems and effects thereof.

Lundgren and Duan (2013) identified other reputed risks to NTOs based on the pharmaceutical literature such as immune stimulation and over-saturation of the

RNAi machinery. However, as discussed by Bachman et al. (2016), the diets of NTOs consist of plant or animal sources which naturally contain dsRNAs, and there exists a long history of safe consumption of these endogenous dsRNA across eukaryotes. With constant oral exposure to environmental dsRNA endogenously present in natural food sources, unintended effects in nontarget organisms from immune stimulation and RNA machinery saturation are extremely unlikely to result from relatively low exposures to dsRNA resulting from cultivation of MON 87411.

The environmental fate of dsRNA can be addressed in terms of stability and persistence in the environment (Heinemann et al. 2011). Recent studies reported by Dubelman et al. (2014) indicate that the biological activity of *Snf7* dsRNA is lost within 2 days after application to different types of soil. Also, up to 90% degradation of the applied dsRNA in soil was observed within 35 h. The other potential route of exposure is through food webs and risks associated can be addressed through experiments with primary consumers and RNAi consumption by the same (Roberts et al. 2015). Resistance evolution in insects to RNAi has not been addressed and documented yet. It is anticipated that insects that carry viruses with RNAi suppressors would be at a selective advantage on RNAi-protected crops, and RNAi-based prophylactics for honey bee colonies would select for viral pathogens with RNAi suppression (Scott et al. 2013). These authors also discuss how genetic variability within and among insect populations, mismatch between dsRNA and target transcript, and single-nucleotide polymorphisms (SNPs) could provide selective advantage for resistance evolution.

Although, the specificity and robustness of RNAi have triggered an immense interest in using RNAi as a tool for creating insect-resistant crops, commercialization is likely to be fraught with challenges. Beyond safety issues, a major impediment includes the lack of a comprehensive federal regulatory framework for estimating the environmental and ecological risks posed by these technologies. Technology evaluation is ongoing, and the development of a standardized risk assessment paradigm is being developed concurrently. However, a number of critical gaps remain including off-target effects, environmental fate, and importantly, the risk of resistance evolution in target pests. Concurrent with limitations such as off-target effects, toxicity, and unsafe delivery methods that have to be overcome before RNAi can be considered for widespread commercial applications in agriculture, it is crucial that a risk assessment paradigm that can proactively anticipate potential nontarget effects be developed for pesticidal RNAs prior to the lifting of deregulation of this technology. Studies reported by Bachman et al. (2016), Ahmad et al. (2016), Pan et al. (2016), and Yang et al. (2016) can potentially form a basis for risk assessment of RNAi-based GM plants or products, within the existing regulatory frameworks.

Conclusions

While significant advances in RNAi methods and applications in agriculture have occurred recently, especially against viruses, the efficacy of the approach against insect pests in the field is yet to be fully established. The discovery of RNAi and the subsequent research on RNAi in insects have demonstrated the

profound impact that this technology can have not only in understanding gene regulation in insects but developing pest management solutions for protecting plants from insect herbivory. The applications of RNAi have been studied in several target insect pests belonging to orders such as Coleoptera, Lepidoptera, and Hemiptera. These studies paved the way to a better understanding of using RNAi as a pest management tool, while concurrently highlighting the caveats of using this tool for sustainable management of crop pests. The experimental data generated in the laboratory on several targets shows that with the exception of few studies, the phenotypes observed were mostly sublethal, and field-efficacy data is lacking for many targets and species. It is now evident that the effects of dsRNA; both target and off-target; are species-dependent and target gene-dependent. RNAi provides a mode of action unique among insecticidal agents through the mechanism of gene suppression and therefore can complement the current methods deployed for pest control. Although the current regulatory system allows following the existing methods, further refinement may be required in terms of measurement of target and off-target effects. Therefore, the future course of action for deploying this technology on a commercial scale depends on how these challenges are addressed. Also, the technologies that enable effective and efficient RNAi sprays such as BioClay, based on nanoparticles, should be further explored.

References

Abrieux A, Debernard S, Maria A, Gaertner C, Anton S, Gadenne C, Duportets L (2013) Involvement of the G-protein-coupled dopamine/ecdysteroid receptor DopEcR in the behavioral response to sex pheromone in an insect. PLoS One 8:88. doi:10.1371/journal.pone.0072785

Adams MD, Celniker SE, Holt RA, Evans CA, Gocayne JD et al (2000) The genome sequence of *Drosophila melanogaster*. Science 287:2185–2195

Agrawal N, Malhotra P, Bhatnagar RK (2004) siRNA-directed silencing of transgene expressed in cultured insect cells. Biochem Biophys Res Commun 320:428–434

Ahmad A, Negri I, Oliveira W, Brown C, Asiimwe P, Sammons B, Horak M, Jiang C, Carson D (2016) Transportable data from non-target arthropod field studies for the environmental risk assessment of genetically modified maize expressing an insecticidal double-stranded RNA. Transgenic Res 25:1–17

Allen ML, Walker WB (2012) Saliva of *Lygus lineolaris* digests double stranded ribonucleic acids. J Insect Physiol 58:391–396. doi:10.1016/j.jinsphys.2011.12.014

Alves AP, Lorenzen MD, Beeman RW, Foster JE, Siegfried BD (2010) RNA interference as a method for target-site screening in the western corn rootworm, *Diabrotica virgifera virgifera*. J Insect Sci 10:162. doi:10.1673/031.010.14122

Andrade CE, Hunter WB (2016) RNA interference – natural gene-based technology for highly specific pest control (HiSPeC). In: Abdurakhmonov IY (ed) RNA interference. InTech, Croatia, pp 391–409

Antonio DSM, Guidugli-Lazzarini KR, Do Nascimento AM, ZLP S, Hartfelder K (2008) RNAi-mediated silencing of vitellogenin gene function turns honeybee (*Apis mellifera*) workers into extremely precocious foragers. Naturwissenschaften 95:953–961. doi:10.1007/s00114-008-0413-9

Arakane Y, Hogenkamp DG, Zhu YC, Kramer KJ, Specht CA, Beeman RW, Kanost MR, Muthukrishnan S (2004) Characterization of two chitin synthase genes of the red flour beetle,

Tribolium castaneum, and alternate exon usage in one of the genes during development. Insect Biochem Mol Biol 34:291–304

Arimatsu Y, Kotani E, Sugimura Y, Furusawa T (2007) Molecular characterization of a cDNA encoding extracellular dsRNase and its expression in the silkworm, *Bombyx mori*. Insect Biochem Mol Biol 37:176–183

Aronstein K, Saldivar E (2005) Characterization of a honey bee Toll related receptor gene *Am18w* and its potential involvement in antimicrobial immune defense. Apidologie 36:3–14

Aronstein K, Oppert B, Lorenzen M (2011) RNAi in agriculturally-important arthropods. In: Grabowski PP (ed) RNA processing. In Tech, Shanghai, pp 157–180

Asgari S (2013) MicroRNA functions in insect. Insect Biochem Mol Biol 43:388–397

Auer C, Frederick R (2009) Crop improvement using small RNAs: applications and predictive ecological risk assessments. Trends Biotechnol 27:644–651. doi:10.1016/j.tibtech.2009.08.005

Bachman PM, Huizinga KM, Jensen PD, Mueller G, Tan J, Uffman JP, Levine SL (2016) Ecological risk assessment for DvSnf7 RNA: a plant-incorporated protectant with targeted activity against western corn rootworm. Regul Toxicol Pharmacol 81:77–88. doi:10.1016/j.yrtph.2016.08.001

Barnard A-C, Nijhof AM, Gaspar ARM, Neitz AWH, Jongejan F, Maritz-Olivier C (2012) Expression profiling, gene silencing and transcriptional networking of metzincin metalloproteases in the cattle tick, *Rhipicephalus (Boophilus) microplus*. Vet Parasitol 186:403–414. doi:10.1016/j.vetpar.2011.11.026

Baum JA, Roberts JK (2014) Progress towards RNAi-mediated insect pest management. In: Dhadialla TS, Gill SS (eds) Insect midgut and insecticidal proteins, Advances in insect physiology, vol 47. Academic, London, pp 249–295

Baum JA, Bogaert T, Clinton W, Heck GR, Feldmann P, Ilagan O et al (2007) Control of coleopteran insect pests through RNA interference. Nat Biotechnol 25:1322–1326. doi:10.1038/nbt1359

Baum JA, Cajacob CA, Feldmann P, Heck GR, Nooren I, Plaetinck G, Maddelein W, Vaughn T (2011) Methods for control of insect infestation in plants and compositions thereof. US Patent No 7, 943,819

Bautista MAM, Miyata T, Miura K, Tanaka T (2009) RNA interference-mediated knockdown of a cytochrome P450, CYP6BG1, from the diamondback moth, *Plutella xylostella*, reduces larval resistance to permethrin. Insect Biochem Mol Biol 39:38–46

Beck M, Strand MR (2005) *Glc1.8* from *Microplitis demolitor* bracovirus induces a loss of adhesion and phagocytosis in insect high five and S2 cells. J Virol 79:1861–1870

Belles X (2010) Beyond *Drosophila*: RNAi in vivo and functional genomics in insects. Annu Rev Entomol 55:111–128

Bettencourt R, Terenius O, Faye I (2002) Hemolin gene silencing by ds-RNA injected into *Cecropia* pupae is lethal to next generation embryos. Insect Mol Biol 11:267–271

Bhatia V, Bhattacharya R, Uniyal PL, Singh R, Niranjan RS (2012) Host generated siRNAs attenuate expression of serine protease gene in *Myzus persicae*. PLoS One 7:e46343. doi:10.1371/journal.pone.0046343

Blandin S, Moita LF, Köcher T, Wilm M, Kafatos FC, Levashina EA (2002) Reverse genetics in the mosquito *Anopheles gambiae*: targeted disruption of the *Defensin* gene. EMBO Rep 3:852–856

Bolognesi R, Ramaseshadri P, Anderson J, Bachman P, Clinton W, Flannagan R et al (2012) Characterizing the mechanism of action of double-stranded RNA activity against western corn rootworm (*Diabrotica virgifera virgifera* LeConte). PLoS One 7:e47534. doi:10.1371/journal.pone.0047534

Borgio JF (2010) RNAi mediated gene knockdown in sucking and chewing insect pests. J Biopesticides 36:153–161

Bronkhorst AW, van Rij RP (2014) The long and short of antiviral defense: small RNA-based immunity in insects. Curr Opin Virol 7:19–28. doi:10.1016/j.coviro.2014.03.010

Camargo Barbosa GO, Possignolo IP et al (2016) RNA interference as a gene silencing tool to control *Tuta absoluta* in tomato (*Solanum lycopersicum*). Peer J 4:e2673. doi:10.7717/peerj.2673

Caplen NJ, Fleenor J, Fire A, Morgan RA (2000) dsRNA-mediated gene silencing in cultured *Drosophila* cells, a tissue culture model for the analysis of RNA interference. Gene 252:95–105

Carrière Y, Crickmore N, Tabashnik BE (2015) Optimizing pyramided transgenic *Bt* crops for sustainable pest management. Nat Biotechnol 33:161–168

Carthew RW, Sontheimer EJ (2009) Origins and mechanisms of miRNAs and siRNAs. Cell 136(4):642–655. doi:10.1016/j.cell.2009.01.035

Casacuberta JM, Devos Y, du Jardin P, Ramon M, Vaucheret H, Nogué F (2014) Biotechnological uses of RNAi in plants: risk assessment considerations. Trends Biotechnol 33:145–147. doi:10.1016/j.tibtech.2014.12.003

Chen J, Zhang D, Yao Q, Zhang J, Dong X, Tian H et al (2010) Feeding-based RNA interference of a trehalose phosphate synthase gene in the brown planthopper, *Nilaparvata lugens*. Insect Mol Biol 19(6):777–786. doi:10.1111/j.1365-2583.2010.01038.x

Chen X, Tian H, Zou L, Tang B, Hu J, Zhang W (2008) Disruption of *Spodoptera exigua* larval development by silencing chitin synthase gene A with RNA interference. Bull Entomol Res 98(06):613–619

Choi H, Glatter T, Gstaiger M, Nesvizhskii AI (2012) SAINT-MS1: protein-protein interaction scoring using label-free intensity data in affinity purification-mass spectrometry experiments. J Proteome Res 11:2619–2624

Christiaens O, Smagghe G (2014) The challenge of RNAi-mediated control of hemipterans. Curr Opin Insect Sci 6:15–21. doi:10.1016/j.cois.2014.09.012

Christiaens O, Sweveres L, Smagghe G (2014) DsRNA degradation in the pea aphid (*Acyrthosiphon pisum*) associated with lack of response in RNAi feeding and injection assay. Peptides 53:307–314. doi:10.1016/j.peptides.2013.12.014

Chu CC, Sun W, Spencer JL, Pittendrigh BR, Seufferheld MJ (2014) Differential effects of RNAi treatments on field populations of the western corn rootworm. Pestic Biochem Physiol 110:1–6

Clemens JC, Worby CA, Simonson-Leff N, Muda M, Maehama T, Hemmings BA, Dixon JE (2000) Use of double-stranded RNA interference in *Drosophila* cell lines to dissect signal transduction pathways. Proc Natl Acad Sci U S A 97:6499–6503

Coleman AD, Wouters RH, Mugford ST, Hogenhout SA (2015) Persistence and transgenerational effect of plant-mediated RNAi in aphids. J Exp Bot 66:541–548

Daly T, Buntin GD (2005) Effects of *Bacillus thuringiensis* transgenic corn for lepidopteran control on non-target arthropods. Environ Entomol 34:1292–1301. doi:10.1603/0046-225X(2005)034[1292:EOBTTC]2.0.CO;2

Dass CR, Choong PF (2008) Chitosan-mediated orally delivered nucleic acids: a gutful of gene therapy. J Drug Target 16:257–261

Desneux N, Luna MG, Guillemaud T, Urbaneja A (2011) The invasive South American tomato pinworm, *Tuta absoluta*, continues to spread in Afro-Eurasia and beyond: the new threat to tomato world production. J Pest Sci 84:403–408

Devos Y, Álvarez-Alfageme F, Gennaro A, Mestdagh S (2015) Assessment of unanticipated unintended effects of genetically modified plants on non-target organisms: a controversy worthy of pursuit? J Appl Entomol 140:1–10. doi:10.1111/jen.12248. [Epub ahead of print]

Dietzl G, Chen D, Schnorrer F, Su KC, Barinova Y, Fellner M, Gasser B, Kinsey K, Oppel S, Scheiblauer S, Couto A, Marra V, Keleman K, Dickson B (2007) A genome-wide transgenic RNAi library for conditional gene inactivation in *Drosophila*. Nature 448:151–156

Dubelman S, Fischer J, Zapata F, Huizinga K, Jiang C, Uffman J et al (2014) Environmental fate of double-stranded RNA in agricultural soils. PLoS One 9:e93155. doi:10.1371/journal.pone.0093155

Dykxhoorn DM, Lieberman J (2005) The silent revolution: RNA interference as basic biology, research tool, and therapeutic. Annu Rev Med 56:401–423

Dzitoyeva S, Dimitrijevic N, Manev H (2001) Intra-abdominal injection of double-stranded RNA into anesthetized adult *Drosophila* triggers RNA interference in the central nervous system. Mol Psychiatry 6(6):665–670

EFSA (European Food Safety Authority) (2010a) EFSA panel on plant protection products and their residues (PPR); scientific opinion on the development of specific protection goal options for environmental risk assessment of pesticides, in particular in relation to the revision of the guidance documents on aquatic and terrestrial ecotoxicology (SANCO/3268/2001 and SANCO/10329/2002). EFSA J 8(10):1821. doi:10.2903/j.efsa.2010.1821. (55 pp)

EFSA (European Food Safety Authority) (2010b) EFSA panel on genetically modified organisms (GMO); scientific opinion on the assessment of potential impacts of genetically modified plants on non-target organisms. EFSA J 8(11):1877. doi:10.2903/j.efsa.2010.1877. (72 pp)

Fabrick JA, Kanost MR, Baker JE (2004) RNAi-induced silencing of embryonic tryptophan oxygenase in the Pyralid moth, *Plodia interpunctella*. J Insect Sci 4(15):9

Farooqui T, Robinson K, Vaessin H, Smith BH (2003) Modulation of early olfactory processing by an octopaminergic reinforcement pathway in the honeybee. J Neurosci 23:5370–5380

Feinberg EH, Hunter CP (2003) Transport of dsRNA into cells by the transmembrane protein SID-1. Science 301:1545–1547

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391:806–811

Fishilevich E, Velez AM, Storer NP, Li HR, Bowling AJ, Rangasamy M, Worden SE, Narva KE, Siegfried BD (2016) RNAi as a management tool for the western corn rootworm, *Diabrotica virgifera virgifera*. Pest Manag Sci 72:1652–1663. doi:10.1002/ps.4324

FSANZ (2013) Response to Heinemann et al. on the regulation of GM crops and foods developed using gene silencing. http://www.foodstandards.govt.nz/consumer/gmfood/Documents/Heinemann%20Response%20210513.pdf

Garbutt JS, Bellés X, Richards EH, Reynolds SE (2013) Persistence of double-stranded RNA in insect hemolymph as a potential determiner of RNA interference success: evidence from *Manduca sexta* and *Blattella germanica*. J Insect Physiol 59:171–178

Gatehouse HS, Gatehouse LN, Malone LA, Hodges S, Tregidga E, Todd J (2004) Amylase activity in honey bee hypopharyngeal glands reduced by RNA interference. J Apic Res 43:9–13

Georghiou GP, Lagunes-Tejeda A (1991) The occurrence of resistance to pesticides in arthropods: an index of cases reported through 1989. Food and Agriculture Organization of the United Nations, Rome

Ghanim M, Kontsedalov S, Czosnek H (2007) Tissue-specific gene silencing by RNA interference in the whitefly, *Bemisia tabaci* (Gennadius). Insect Biochem Mol Biol 37:732–738. doi:10.1016/j.ibmb.2007.04.006

Ghildiyal M, Zamore PD (2009) Small silencing RNAs: an expanding universe. Nat Rev Genet 10:94–108

Gong L, Chen Y, Hu Z, Hu MY (2013) Testing insecticidal activity of novel chemically synthesized siRNA against *Plutella xylostella* under laboratory and field conditions. PLoS One 8:88. doi:10.1371/journal.pone.0062990

Gong LA, Yang XQ, Zhang BL, Zhong GH, Hu MY (2011) Silencing of Rieske iron-sulfur protein using chemically synthesised siRNA as a potential biopesticide against *Plutella xylostella*. Pest Manag Sci 67:514–520. doi:10.1002/ps.2086

Gordon KH, Waterhouse PM (2007) RNAi for insect-proof plants. Nat Biotechnol 25:1231–1232

Hammond SM, Boettcher S, Caudy AA, Kobayashi R, Hannon GJ (2001) Argonaute2, a link between genetic and biochemical analyses of RNAi. Science 293:1146–1150

Hassanien ITE, Meyering-Vos M, Hoffmann KH (2014) RNA interference reveals allatotropin functioning in larvae and adults of *Spodoptera frugiperda* (Lepidoptera, Noctuidae). Entomologia 2:56–64. doi:10.4081/entomologia.2014.169

He B, Chu Y, Yin M, Müllen K, An C, Shen J (2013) Fluorescent nanoparticle delivered dsRNA toward genetic control of insect pests. Adv Mater Weinheim 25:4580–4584. doi:10.1002/adma.201301201

Head G, Moar M, Eubanks M, Freeman B, Ruberson J, Hagerty A, Turnipseed S (2005) A multi-year, large-scale comparison of arthropod populations on commercially managed Bt and non-Bt cotton fields. Environ Entomol 34:1257–1266

Heinemann JA, Kurenbach B, Quist D (2011) Molecular profiling – a tool for addressing emerging gaps in the comparative risk assessment of GMOs. Environ Int 37:1285–1293

Horn T, Boutros M (2010) E-RNAi: a web application for the multi-species design of RNAi reagents – 2010 update. Nucleic Acids Res 38:W332–W339

Horn T, Sandmann T, Boutros M (2010) Design and evaluation of genome-wide libraries for RNA interference screens. Genome Biol 11:R61

Horwich MD, Li C, Matranga C, Vagin V, Farley G, Wang P, Zamore PD (2007) The *Drosophila* RNA methyltransferase, DmHen1, modifies germline piRNAs and single-stranded siRNAs in RISC. Curr Biol 17:1265–1272

Hu X, Richtman NM, Zhao J-Z, Duncan KE, Niu X, Procyk LA, Oneal MA, Kernodle BM, Steimel JP, Crane VC, Sandahl G, Ritland JL, Howard RJ, Presnail JK, Lu AL, Wu G (2016) Discovery of midgut genes for the RNA interference control of corn rootworm. Sci Rep 6:30542. doi:10.1038/srep30542

Huang JH, Lee HJ (2011) RNA interference unveils functions of the hypertrehalosemic hormone on cyclic fluctuation of hemolymph trehalose and oviposition in the virgin female *Blatella germanica*. J Insect Physiol 57:858–864

Hunter W, Ellis J, Vanengelsdorp D, Hayes J, Westervelt D, Glick E, Williams M, Sela I, Maori E, Pettis J et al (2010) Large-scale field application of RNAi technology reducing Israeli acute paralysis virus disease in honey bees (*Apis mellifera*, Hymenoptera: Apidae). PLoS Pathog 6:e1001160

Hunter WB, Glick E, Paldi N, Bextine BR (2012) Advances in RNA interference: dsRNA treatment in trees and grapevines for insect pest suppression. Southwest Entomol 37:85–87. doi:10.3958/059.037.0110

Huvenne H, Smagghe G (2010) Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: a review. J Insect Physiol 56(3):227–235. doi:10.1016/j.jinsphys.2009.10.004

ILSI-CERA (2011) Problem formulation for the environmental risk assessment of RNAi plants. International Life Sciences Institute, Center for Environmental Risk Assessment, Washington, D.C.

Ivashuta S, Zhang Y, Wiggins BE, Ramaseshadri P, Segers GC, Johnson S et al (2015) Environmental RNAi in herbivorous insects. RNA 21:840–850. doi:10.1261/rna.048116.114

James C (2014) Global status of commercialized biotech/GM Crops: 2014. ISAAA Brief No. 49. ISAAA, Ithaca, NY

Jaubert-Possamai S, Le T, Bonhomme G, Christophides J, Rispe GK, Tagu D (2007) Gene knockdown by RNAi in the pea aphid *Acyrthosiphon pisum*. BMC Biotechnol 7:63

Joga MR, Zotti MJ, Smagghe G, Christiaens O (2016) RNAi efficiency, systemic properties, and novel delivery methods for pest insect control: what we know so far. Front Physiol 7:553. doi:10.3389/fphys.2016.00553

Jose AM, Hunter CP (2007) Transport of sequence-specific RNA interference information between cells. Annu Rev Genet 41:305–330

Kamath RS, Martinex-Campos M, Zipperlen P, Frasher AG, Ahringer J (2000) Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in *Caenorhabditis elegans*. Genome Biol 2:research0002

Katoch R, Sethi A, Thakur N, Murdock LL (2013) RNAi for insect control: current perspective and future challenges. Appl Biochem Biotechnol 171(4):847–873

Kemp C, Mueller S, Goto A, Barbier V, Paro S, Bonnay F, Dostert C, Troxler L, Hetru C, Meignin C, Pfeffer S, Hoffmann JA, Imler JL (2013) Broad RNA interference-mediated antiviral immunity and virus-specific inducible responses in *Drosophila*. J Immunol 190:650–658

Kennerdell JR, Carthew RW (1998) Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled 2 act in the wingless pathway. Cell 95:1017–1026

Khajuria C, Buschman LL, Chen MS, Muthukrishnan S, Zhu KY (2010) A gut-specific chitinase gene essential for regulation of chitin content of peritrophic matrix and growth of *Ostrinia nubilalis* larvae. Insect Biochem Mol Biol 40:621–629. doi:10.1016/j.ibmb.2010.06.003

Khajuria C, Li H, Narva K, Rangasamy M, Siegfried B (2013) Effectiveness of dsRNA versus siRNA in RNAi mediated gene knock-down in western corn rootworm (*Diabrotica virgifera virgifera*). In: Program and abstracts, 46th annual meeting of the Society for Invertebrate Pathology Conference on Invertebrate Pathology and Microbial Control. Society for Invertebrate Pathology. Pittsburgh, PA

Khajuria C, Vélez AM, Rangasamy M, Wang H, Fishilevich E, Frey MLF, Carneiro N, Premchand G, Narva KE, Siegfried BD (2015) Parental RNA interference of genes involved in embryonic

development of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte. Insect Biochem Mol Biol 63:54–62

Kitzmann P, Schwirz J, Schmitt-Engel C, Bucher G (2013) RNAi phenotypes are influenced by the genetic background of the injected strain. BMC Genomics 14(1):5. doi:10.1186/1471-2164-14-5

Koch A, Kogel KH (2014) New wind in the sails: improving the agronomic value of crop plants through RNAi-mediated gene silencing. Plant Biotechnol J 12:821–831. doi:10.1111/pbi.12226

Kolliopoulou A, Swevers L (2014) Science direct recent progress in RNAi research in lepidoptera?: intracellular machinery, antiviral immune response and prospects for insect pest control. Curr Opin Insect Sci:1–7. doi:10.1016/j.cois.2014.09.019

Kontogiannatos D, Swevers L, Maenaka K, Park EY, Iatrou K, Kourti A (2013) A functional characterization of a Juvenile Hormone esterase related gene in the moth *Sesamia nonagrioides* through RNA interference. PLoS One 8:88. doi:10.1371/journal.pone.0073834

Kumar M, Gupta GP, Rajam MV (2009) Silencing of acetylcholinesterase gene of *Helicoverpa armigera* by siRNA affects larval growth and its life cycle. J Insect Physiol 55:273–278. doi:10.1016/j.jinsphys.2008.12.005

Ladics GS, Bartholomaeus A, Bregitzer P, Doerrer NG, Gray A, Holzhauser T et al (2015) Genetic basis and detection of unintended effects in genetically modified crop plants. Transgenic Res 24:587–603. doi:10.1007/s11248-015-9867-7

Lawo NC, Wackers FL, Romeis J (2009) Indian Bt cotton varieties do not affect the performance of cotton aphids. PLoS One 4:e4804

Lee YS, Nakahara K, Pham JW, Kim K, He Z, Sontheimer EJ, Carthew RW (2004) Distinct roles for Drosophila Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. Cell 117:69–81

Li X, Zhang M, Zhang H (2011) RNA interference of four genes in adult *Bactrocera dorsalis* by feeding their dsRNAs. PLoS One 6(3):e17788. doi:10.1371/journal.pone

Li Y, Romeis J (2010) *Bt* maize expressing Cry3Bb1 does not harm the spider mite, *Tetranychus urticae*, or its ladybird beetle predator, *Stethorus punctillum*. Biol Control 53:337–344. doi:10.1016/j.biocontrol.2009.12.003

Liu F, Wang X, Zhao Y, Li Y, Liu Y, Sun J (2015) Silencing the HaAK gene by transgenic plant-mediated RNAi impairs larval growth of *Helicoverpa armigera*. Int J Biol Sci 11:67–74. doi:10.7150/ijbs.10468

Liu S, Ding Z, Zhang C, Yang B, Liu Z (2010) Gene knockdown by introthoracic injection of double-stranded RNA in the brown planthopper, *Nilaparvata lugens*. Insect Biochem Mol Biol 40:666–671

Liu Y, Ye X, Jiang F, Liang C, Chen D, Peng J, Kinch LN, Grishin NV, Liu Q (2009) C3PO, an endoribonuclease that promotes RNAi by facilitating RISC activation. Science 325:750–753

Loy DL, Mogler MA, Loy DS, Janke B, Kamrud K, Scura ED, Harris DLH, Bartholomay LC (2012) Double-stranded RNA provides sequence dependent protection against infectious myonecrosis virus in *Litopenaeus vannamei*. J Gen Virol 93:880–888

Lum L, Yao S, Mozer B, Rovescalli A, Von Kessler D, Nirenberg M, Beachy PA (2003) Science 299:2039–2045

Lundgren JG, Duan JJ (2013) RNAi-based insecticidal crops. Bioscience 63:657–665. doi:10.1525/bio.2013.63.8.8

Luo Y, Wang X, Yu D, Kang L (2012) The SID-1 double-stranded RNA transporter is not required for systemic RNAi in the migratory locust. RNA Biol 9:663–671

Mamta RKR, Rajam MV (2016) Targeting chitinase gene of *Helicoverpa armigera* by host-induced RNA interference confers insect resistance in tobacco and tomato. Plant Mol Biol 90:281–292. doi:10.1007/s11103-015-0414-y

Mao J, Zeng F (2012) Feeding-based RNA interference of a gap gene is lethal to the pea aphid, Acyrthosiphon pisum. PLoS One 7:e48718

Mao J, Zeng F (2014) Plant-mediated RNAi of a gap gene-enhanced tobacco tolerance against the *Myzus persicae*. Transgenic Res 23:145–152. doi:10.1007/s11248-013-9739-y

Mao YB, Cai WJ, Wang JW, Hong GJ, Tao XY, Wang LJ, Huang YP, Chen XY (2007) Silencing a cotton bollworm P450 monooxygenase gene by plant-mediated RNAi impairs larval tolerance of gossypol. Nat Biotechnol 25(11):1307–1313

Mao YB, Tao XY, Xue XY, Wang LJ, Chen XY (2011) Cotton plants expressing *CYP6AE14* double-stranded RNA show enhanced resistance to bollworms. Transgenic Res 20:665–673. doi:10.1007/s11248-010-9450-1

Mao YB, Xue XY, Tao XY, Yang CQ, Wang LJ, Chen XY (2013) Cysteine protease enhances plant-mediated bollworm RNA interference. Plant Mol Biol 83:119–129. doi:10.1007/s11103-013-0030-7

Martin D, Maestro O, Cruz J, Mane-Padros D, Belles X (2006) RNAi studies reveal a conserved role for RXR in molting in the cockroach *Blattella germanica*. J Insect Physiol 52:410–416

Meister G, Tuschl T (2004) Mechanisms of gene silencing by double-stranded RNA. Nature 431(7006):343–349

Miguel SK, Scott JG (2016) The next generation of insecticides: dsRNA is stable as a foliar-applied insecticide. Pest Manag Sci 72(4):801–809. doi:10.1002/ps.4056

Miller SC, Brown SJ, Tomoyasu Y (2008) Larval RNAi in *Drosophila*? Dev Genes Evol 218:505–510

Mitter N, Elizabeth AW, Karl ER, Li P, Jain RG et al (2017) Clay nanosheets for topical delivery of RNAi for sustained protection against plant viruses. Nature Plants 3:16207. doi:10.1038/nplants.2016.207

Mohammed A, Diab MR, Abd-Alla SMM, Hussien EHA (2015) RNA interference – mediated knockdown of vacuolar ATPase genes in pink bollworm, *Pectinophora gossypiella*. Int J Biol Pharm Allied Sci 4:2641–2660

Mohr S, Perrimon N (2012) RNAi screening: new approaches, understandings and organisms. Wiley Interdiscip Rev RNA 2:145–158

Mutti NS, Park Y, Reese JC, Reeck GR (2006) RNAi knockdown of a salivary transcript leading to lethality in the pea aphid, *Acyrthosiphon pisum*. J Insect Sci 6:1–7. doi:10.1673/031.006.3801

Mutti NS, Louis J, Pappan LK, Pappan K, Begum K, Chen MS, Park Y, Dittmer N, Marshall J, Reese JC, Reeck GR (2008) A protein from the salivary glands of the pea aphid, *Acyrthosiphon pisum*, is essential in feeding on a host plant. Proc Natl Acad Sci U S A 105:9965–9969

Mysore K, Andrews E, Li P, Duman-Scheel M (2014) Chitosan/siRNA nanoparticle targeting demonstrates a requirement for single-minded during larval and pupal olfactory system development of the vector mosquito *Aedes aegypti*. BMC Dev Biol 14:1. doi:10.1186/1471-213X-14-9

Naito Y, Yamuda T, Mastumiya T, Kumiko UT, Saigo K, Morishita S (2005) dsCheck: highly sensitiveoff-target search software for double-stranded RNA-mediated RNA interference. Nucleic Acids Res 33:W589–W591

Nakamura T, Mito T, Miyawaki K, Ohuchi H, Noji S (2008) EGFR signaling is required for re-establishing the proximodistal axis during distal leg regeneration in the cricket *Gryllus bimaculatus* nymph. Dev Biol 319:46–55

Naranjo SE (2005) Long-term assessment of the effects of transgenic Bt cotton on the abundance of nontarget arthropod natural enemies. Environ Entomol 34:1193–1210

Nayak A, Tassetto M, Kunitomi M, Andino R (2013) RNA interference-mediated intrinsic antiviral immunity in invertebrates. Curr Top Microbiol Immunol 371:183–200

Osanai-Futahashi M, Tatematsu K-I, Futahashi R, Narukawa J, Takasu Y, Kayukawa T, Shinoda T, Ishige T, Yajima S, Tamura T, Yamamoto K, Sezutsu H (2016) Positional cloning of a *Bombyx* pink-eyed white egg locus reveals the major role of cardinal in ommochrome synthesis. Heredity 116:135–145. doi:10.1038/hdy.2015.74

Paim RM, Araujo RN, Lehane MJ, Gontijo NF, Pereira MH (2013) Long-term effects and parental RNAi in the blood feeder, *Rhodnius prolixus* (Hemiptera; Reduviidae). Insect Biochem Mol Biol 43:1015–1020. doi:10.1016/j.ibmb.2013.08.008

Palli SR (2012) RNAi methods for management of insects and their pathogens. CAB Rev 7:1–10

Palli SR (2014) RNA interference in Colorado potato beetle: steps toward development of dsRNA as a commercial insecticide. Curr Opin Insect Sci 6:1–8. doi:10.1016/j.cois.2014.09.011

Pan H, Xu L, Noland JE, Li H, Siegfried BD, Zhou X (2016) Assessment of potential risks of dietary RNAi to a soil micro-arthropod, *Sinella curviseta* Brook (Collembola: Entomobryidae). Front Plant Sci 7:1028. doi:10.3389/fpls.2016.01028

Perrimon N, Mathey-Prevot B (2007) Applications of high-throughput RNA interference screens to problems in cell and developmental biology. Genetics 175:7–16

Peters L, Meister G (2007) Argonaute proteins: mediators of RNA silencing. Mol Cell 26(5):611–623
Pitino M, Coleman AD, Maffei ME, Ridout CJ, Hogenhout SA (2011) Silencing of aphid genes by dsRNA feeding from plants. PLoS One 6:e25709
Pradhan SK, Nayak DK, Mohanty S, Behera L, Barik SR, Pandit E, Lenka S, Anandan A (2015) Pyramiding of three bacterial blight resistance genes for broad-spectrum resistance in deepwater rice variety, Jalmagna. Rice 8:19. doi:10.1186/s12284-015-0051-8
Price DRG, Gatehouse JA (2008) RNAi-mediated crop protection against insects. Trends Biotechnol 26(7):393–400
Pridgeon JW, Zhao L, Becnel JJ, Strickman DA, Clark GG, Linthicum KJ (2008) Topically applied AaeIAP1 double-stranded RNA kills female adults of *Aedes aegypti*. J Med Entomol 45:414–420
Qi XL, Su XF, Lu GQ, Liu CX, Liang GM, Cheng HM (2015) The effect of silencing arginine kinase by RNAi on the larval development of *Helicoverpa armigera*. Bull Entomol Res 105:555–565. doi:10.1017/S0007485315000450
Qian D, Tian L, Qu L (2015) Proteomic analysis of endoplasmic reticulum stress responses in rice seeds. Sci Rep 5:14255. doi:10.1038/srep14255
Qiu S, Adema CM, Lane T (2005) A computational study of off-target effects of RNA interference. Nucleic Acids Res 33:1834–1847
Quan GX, Kanda T, Tamura T (2002) Induction of the *white* egg 3 mutant phenotype by injection of the double-stranded RNA of the silkworm *white* gene. Insect Mol Biol 11:217–222
Rajagopal S, Sivakumar N, Agrawal P, Malhotra P, Bhatnagar RK (2002) Silencing of midgut aminopeptidase N of *Spodoptera litura* by double-stranded RNA establishes its role as *Bacillus thuringiensis* toxin receptor. J Biol Chem 277:46849–46851
Ramon M, Devos Y, Lanzoni A, Liu Y, Gomes A, Gennaro A et al (2014) RNAi-based GM plants: food for thought for risk assessors. Plant Biotechnol J 12:1271–1273. doi:10.1111/pbi.12305
Rangasamy M, Siegfried BD (2012) Validation of RNA interference in western corn rootworm *Diabrotica virgifera virgifera* LeConte (Coleoptera, Chrysomelidae) adults. Pest Manag Sci 68:587–591. doi:10.1002/ps.2301
Ratzka C, Gross R, Feldhaa H (2013) Systemic gene knockdown in *Camponotus floridanus* workers by feeding of dsRNA. Insect Soc 60(4):475–484. doi:10.1007/s00040-013-0314-6
Raybould A (2011) The bucket and the searchlight: formulating and testing risk hypotheses about the weediness and invasiveness potential of transgenic crops. Environ Biosaf Res 9:123–133
Rinkevich FD, Scott JG (2013) Limitations of RNAi of a6 nicotinic acetylcholine receptor subunits for assessing the in vivo sensitivity to spinosad. Insect Sci 20:101–108
Roberts AF, Devos Y, Lemgo GNY, Zhou X (2015) Biosafety research for non-target organism risk assessment of RNAi-based GE plants. Front Plant Sci 6:958. doi:10.3389/fpls.2015.00958
Rodrigues TB, Figueira A (2016) Management of insect pest by RNAi – a new tool for crop protection. In: Abdurakhmonov IY (ed) RNA interference. InTech, Croatia. doi:10.5772/61807
Rodriguez CL, Trujillo BD, Borras HO, Wright DJ, Ayra-Pardo C (2010) RNAi-mediated knockdown of a *Spodoptera frugiperda* trypsin-like serine-protease gene reduces susceptibility to a *Bacillus thuringiensis* Cry1Ca1 protoxin. Environ Microbiol 12:2894–2903. doi:10.1111/j.1462-2920.2010.02259.x
Roignant JY, Carré C, Mugat B, Szymczak D, Lepesant JA, Antoniewski C (2003) Absence of transitive and systemic pathways allows cell-specific and isoform-specific RNAi in *Drosophila*. RNA 9:299–230
Romeis J, Bartsch D, Bigler F, Candolfi MP, Gielkens MMC et al (2008) Assessment of risk of insect-resistant transgenic crops to nontarget arthropods. Nat Biotechnol 26(2):203–208. doi:10.1038/nbt1381
Romeis J, Hellmich RL, Candolfi MP, Carstens K, De Schrijver A et al (2011) Recommendations for the design of laboratory studies on non-target arthropods for risk assessment of genetically engineered plants. Transgenic Res 20:1–22. doi:10.1007/s11248-010-9446-x

Romeis J, Raybould A, Bigler F, Candolfi MP, Hellmich RL, Huesing JE, Shelton AM (2013) Deriving criteria to select arthropod species for laboratory tests to assess the ecological risks from cultivating arthropod-resistant genetically engineered crops. Chemosphere 90(2013):901–909

Rose RI (ed) (2007) White paper on tier-based testing for the effects of proteinaceous insecticidal plant-incorporated protectants on non-target invertebrates for regulatory risk assessment. USDA-APHIS and US Environmental Protection Agency, Washington, DC. http://www.epa.gov/pesticides/biopesticides/pips/non-target-arthropods.pdf

Sadeghi A, van Damme EJM, Smagghe G (2009) Evaluation of the susceptibility of the pea aphid, *Acyrthosiphon pisum*, to a selection of novel biorational insecticides via artificial diet. J Insect Sci 9:65. http://insectscience.org/9.65

Saito K, Sakaguchi Y, Suzuki T, Suzuki T, Siomi H, Siomi MC (2007) Pimet, the *Drosophila* homolog of HEN1, mediates 2′-*O*-methylation of Piwi- interacting RNAs at their 3′ ends. Genes Dev 21:1603–1608

Saleh MC, van Rij RP, Hekele A, Gillis A, Foley E, O'Farrell PH et al (2006) The endocytic pathway mediates cell entry of dsRNA to induce RNAi silencing. Nat Cell Biol 8:793–802. doi:10.1038/ncbl439

Sarkies P, Miska EA (2013) Is there social RNA? Science 341(6145):467–468. doi:10.1126/science.1243175

Schluns H, Crozier RH (2007) Relish regulates expression of antimicrobial peptide genes in the honeybee, *Apis mellifera*, shown by RNA interference. Insect Mol Biol 16:753–759

Schnell J, Steele M, Bean J, Neuspiel M, Girard C, Dormann N et al (2015) A comparative analysis of insertional effects in genetically engineered plants: considerations for pre-market assessments. Transgenic Res 24:1–17. doi:10.1007/s11248-014-9843-7

Scott JG, Michel K, Bartholomay LC, Siegfried BD, Hunter WB, Smagghe G, Zhu KY, Douglas AE (2013) Towards the elements of successful insect RNAi. J Insect Physiol 59:1212–1221. doi:10.1016/j.jinsphys.2013.08.014

Shah C, Förstemann K (2008) Monitoring miRNA mediated silencing in *Drosophila melanogaster* S2-cells. Biochim Biophys Acta 1779:766–772

Shakesby A, Wallace I, Isaacs H, Pritchard J, Roberts D, Douglas A (2009) A water-specific aquaporin involved in aphid osmoregulation. Insect Biochem Mol Biol 39:1–10. doi:10.1016/j.ibmb.2008.08.008

Shukla JN, Kalsi M, Sethi A, Narva KE, Fishilevich E, Singh S et al (2016) Reduced stability and intracellular transport of dsRNA contribute to poor RNAi response in lepidopteran insects. RNA Biol 13(7):656–669. doi:10.1080/15476286.2016.1191728

Sijen T, Fleenor J, Simmer F, Thijssen KL, Parrish S, Timmons L, Plasterk RH, Fire A (2001) On the role of RNA amplification in dsRNA-triggered gene silencing. Cell 107:465–476

Singh AD, Wong S, Ryan CP, Whyard S (2013) Oral delivery of double stranded RNA in larvae of the yellow fever mosquito, *Aedes aegypti*: implications for pest mosquito control. J Insect Sci 13:1–18

Siomi H, Siomi MC (2009) On the road to reading the RNA-interference code. Nature 457:396–404

Sivakumar S, Rajagopal R, Venkatesh GR, Srivastava A, Bhatnagar RK (2007) Knockdown of aminopeptidase-N from *Helicoverpa armigera* larvae and in transfected Sf21 cells by RNA interference reveals its functional interaction with *Bacillus thuringiensis* insecticidal protein Cry1Ac. J Biol Chem 282:7312–7319

Smith P (2013) Delivering food security without increasing pressure on land. Glob Food Sec 2(1):18–23

Soares CAG, Lima CMR, Dolan MC, Piesman J, Beard CB, Zeidner NS (2005) Capillary feeding of specific dsRNA induces silencing of the isac gene in nymphal *Ixodes scapularis* ticks. Insect Mol Biol 14:443–452

Storer NP, Babcock JM, Schlenz M, Meade T, Thompson GD, Bing JW, Huckaba RM (2010) Discovery and characterization of field resistance to Bt maize: *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Puerto Rico. J Econ Entomol 103(4):1031–1038

Sugimoto A (2004) High-throughput RNAi in *Caenorhabditis elegans*, genome-wide screens and functional genomics. Differentiation 72:81–91

Sun K, Wolters AMA, Loonen AEHM, Huibers RP, van der Vlugt R, Goverse A, Jacobsen E, Visser RGF, Bai Y (2016) Down-regulation of *Arabidopsis DND1* orthologs in potato and tomato leads to broad-spectrum resistance to late blight and powdery mildew. Transgenic Res 25:123–138. doi:10.1007/s11248-015-9921-5

Surakasi VP, Mohamed AAM, Kim Y (2011) RNA interference of beta 1 integrin subunit impairs development and immune responses of the beet armyworm, *Spodoptera exigua*. J Insect Physiol 57:1537–1544

Suzuki Y, Truman JW, Riddiford LM (2008) The role of Broad in the development of *Tribolium castaneum*: implications for the evolution of the holometabolous insect pupa. Development 135:569–577. doi:10.1242/dev.015263

Swevers L, Smagghe G (2012) Use of RNAi for control of insect crop pests. In: Smagghe G, Diaz I, editors. Arthropod-plant interactions: novel insights and approaches for IPM. Dordrecht: Springer; pp. 177–197.

Swevers L, Broeck JV, Smagghe G (2013) The possible impact of persistent virus infection on the function of the RNAi machinery in insects: a hypothesis. Front Physiol 4:319. doi:10.3389/fphys.2013.00319

Szittya G, Burgyan J (2013) RNA interference-mediated intrinsic antiviral immunity in plants. Curr Top Microbiol Immunol 371:153–181

Tabara H, Grishok A, Mello CC (1998) RNAi in C. elegans: soaking in the genome sequence. Science 282:430–431

Tabashnik BE, Thierry B, Yves C (2013) Insect resistance to Bt crops: lessons from the first billion acres. Nat Biotechnol 31:510–521

Tan EL, Tan TM, Chow VTK, Poh CL (2008) Inhibition of enterovirus 71 in virus-infected mice by RNA interference. Mol Ther 15:1931–1938

Terenius O, Papanicolaou A, Garbutt JS, Eleftherianos I, Huvenne H, Kanginakudru S et al (2011) RNA interference in Lepidoptera: an overview of successful and unsuccessful studies and implications for experimental design. J Insect Physiol 57(2):231–245. doi:10.1016/j.jinsphys.2010.11.006

Thakur N, Upadhyay SK, Verma PC, Chandrashekar K, Tuli R, Singh PK (2014) Enhanced whitefly resistance in transgenic tobacco plants expressing double stranded RNA of v-ATPase a gene. PLoS One 9:88. doi:10.1371/journal.pone.0087235

Tian H, Peng H, Yao Q, Chen H, Xie Q, Tang B et al (2009) Developmental control of a lepidopteran pest *Spodoptera exigua* by ingestion of bacteria expressing dsRNA of a non-midgut gene. PLoS One 4(7):e6225. doi:10.1371/journal.pone.0006225

Timmon L, Fire A (1998) Specific interference by ingested dsRNA. Nature 395:854

Timmons L, Court DL, Fire A (2001) Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*. Gene 263:103–112

Tomizawa M, Noda H (2013) High mortality caused by high dose of dsRNA in the green rice leafhopper, *Nephotettix cincticeps* (Hemiptera: Cicadellidae). Appl Entomol Zool 48:553–559

Tomoyasu Y, Denell RE (2004) Larval RNAi in *Tribolium* (Coleoptera) for analyzing adult development. Dev Genes Evol 214:575–578

Tomoyasu Y, Miller SC, Tomita S, Schoppmeier M, Grossmann D et al (2008) Exploring systemic RNA interference in insects: a genome-wide survey for RNAi genes in *Tribolium*. Genome Biol 9:R10. doi:10.1186/gb-2008-9-1-r10

Torres JB, Ruberson JR (2005) Canopy- and ground- dwelling predatory arthropods in commercial Bt and non-Bt cotton fields: patterns and mechanisms. Environ Entomol 34:1242–1256

Torres JB, Ruberson JR (2007) Abundance and diversity of ground-dwelling arthropods of pest management importance in commercial Bt and non-Bt cotton fields. Ann Appl Biol 150:27–39

Turner CT, Davy MW, MacDiarmid RM, Plummer KM, Birch NP, Newcomb RD (2006) RNA interference in the light brown apple moth, *Epiphyas postvittana* (Walker) induced by double-stranded RNA feeding. Insect Mol Biol 15:383–391

Ulrich J, Dao VA, Majumdar U, Schmitt-Engel C, Schwirz J, Schultheis D et al (2015) Large scale RNAi screen in *Tribolium* reveals novel target genes for pest control and the proteasome as prime target. BMC Genomics 16:674. doi:10.1186/s12864-015-1880-y

Ulvila J, Parikka M, Kleino A, Sormunen R, Ezekowitz RA, Kocks C et al (2006) Double-stranded RNA is internalized by scavenger receptor-mediated endocytosis in Drosophila S2 cells. J Biol Chem 281:14370–14375. doi:10.1074/jbc.M513868200

Upadhyay SK, Chandrashekar K, Thakur N, Verma PC, Borgio JF, Singh PK et al (2011) RNA interference for the control of whiteflies (*Bemisia tabaci*) by oral route. J Biosci 36:153–161. doi:10.1007/s12038-011-9009-1

US EPA (2007) White paper on tier-based testing for the effects of proteinaceous insecticidal plant-incorporated protectants on non-target arthropods for regulatory risk assessments. United States Environmental Protection Agency, Washington, DC

US EPA (2014) Scientific advisory panel minutes No. 2014-02 (Arlington, VA), 1–77. Available online at: http://www.epa.gov/scipoly/sap/meetings/2014/january/012814minutes.pdf

Valdes VJ, Sampieri A, Sepulveda J, Vaca L (2003) Using double-stranded RNA to prevent in vitro and in vivo viral infections by recombinant baculovirus. J Biol Chem 278:19317–19324

Valdes VJ, Athie A, Salinas LS, Navarro RE, Vaca L (2012) Correction: CUP-1 is a novel protein involved in dietary cholesterol uptake in *Caenorhabditis elegans*. PLoS One 7(8). doi:10.1371/annotation/5a203055-6c15-43b0-96ad-0fbd5eb9b810

Vélez AM, Fishilevich E, Matz N, Storer NP, Narva KE, Siegfried BD (2017) Parameters for successful parental RNAi as an insect pest management tool in western corn rootworm, *Diabrotica virgifera virgifera*. Genes 8:7. doi:10.3390/genes8010007

Vijayendran D, Airs PM, Dolezal K, Bonning BC (2013) Arthropod viruses and small RNAs. J Invertebr Pathol 114(2):186–195

Walker WB, Allen ML (2011) RNA interference-mediated knockdown of IAP in *Lygus lineolaris* induces mortality in adult and preadult life stages. Entomol Exp Appl 138:83–92. doi:10.1111/j.1570-7458.2010.01078.x

Wang Y, Zhang H, Li H, Miao X (2011) Second-generation sequencing supply an effective way to screen RNAi targets in large scale for potential application in pest insect control. PLoS One 6:e18644

Whangbo JS, Hunter CP (2008) Environmental RNA interference. Trends Genet 24(6):297–305. doi:10.1016/j.tig.2008.03.007

Whitten MMA, Facey PD, Del Sol R, Fernández-Martínez LT, Evans MC, Mitchell JJ et al (2016) Symbiont-mediated RNA interference in insects. Proc Biol Sci 283:20160042. doi:10.1098/rspb.2016.0042

Whyard S, Singh AD, Wong S (2009) Ingested double-stranded RNAs can act as species-specific insecticides. Insect Biochem Mol Biol 39:824–832. doi:10.1016/j.ibmb.2009.09.007

Winston WM, Molodowitch C, Hunter CP (2002) Systemic RNAi in *C. elegans* requires the putative transmembrane protein SID-1. Science 295:2456–2459. doi:10.1126/science.1068836

Wu F, Wang PY, Zhao QL, Kang LQ, Xia DG, Qiu ZY, Tang SM, Li MW, Shen XJ, Zhang GZ (2016) Mutation of a cuticle protein gene, BmCPG10, is responsible for silkworm non-moulting in the 2nd instar mutant. PLoS One 11:88. doi:10.1371/journal.pone.0153549

Wuriyanghan H, Rosa C, Falk BW (2011) Oral delivery of double-stranded RNAs and siRNAs induces RNAi effects in the potato/tomato psyllid, *Bactericerca cockerelli*. PLoS One 6:e27736

Xiong Y, Zeng H, Zhang Y, Xu D, Qiu D (2013) Silencing the HaHR3 Gene by transgenic plant-mediated RNAi to disrupt *Helicoverpa armigera* development. Int J Biol Sci 9:370–381. doi:10.7150/ijbs.5929

Xu J, Wang X-F, Chen P, Liu FT, Zheng SC, Ye H, Mo MH (2016) RNA interference in moths: mechanisms, applications and progress. Genes 7:88. doi:10.3390/genes7100088

Xu W, Han Z (2008) Cloning and phylogenetic analysis of sid-1-like genes from aphids. J Insect Sci 8:30. doi:10.1673/031.008.3001

Xue XY, Mao YB, Tao XY, Huang YP, Chen XY (2012) New approaches to agricultural insect pest control based on RNA interference. Adv Insect Physiol 42:73–117

Yan T, Chen H, Sun Y, Yu X, Xia L (2016) RNA interference of the ecdysone receptor genes *EcR* and *USP* in grain aphid (*Sitobion avenae* F.) affects its survival and fecundity upon feeding on wheat plants. Davies TGE, ed. Int J Mol Sci 17(12):2098. doi:10.3390/ijms17122098

Yang C, Preisse EL, Zhang H, Liu Y, Dai L, Pan H, Pan H, Zhou X (2016) Selection of reference genes for RT-qPCR analysis in *Coccinella septempunctata* to assess un-intended effects of RNAi transgenic plants. Front Plant Sci 7:1672. doi:10.3389/fpls.2016.01672

Yang J, Han ZJ (2014) Efficiency of different methods for dsRNA delivery in cotton bollworm (*Helicoverpa armigera*). J Integr Agric 13:115–123. doi:10.1016/S2095-3119(13)60511-0

Yao J, Rotenberg D, Afsharifar A, Barandoc AK, Whitfield AE (2013) Development of RNAi methods for *Peregrinus maidis*, the corn planthopper. PLoS One 8:e70243

Yu N, Christiaens O, Liu JM, Niu J, Cappelle K, Caccia S, Huvenne H, Smagghe G (2013) Delivery of dsRNA for RNAi in insects: an overview and future directions. Insect Sci 20:4–14

YuQ LT, Feng G, Yang K, Pang Y (2008) Functional analysis of the putative antiapoptotic genes, *p49* and *iap4*, of *Spodoptera litura* nucleopolyhedrovirus with RNAi. J Gen Virol 89:1873–1880

Zamore PD (2001) RNA interference: listening to the sound of silence. Nat Struct Mol Biol 8(9):746–750

Zeynep A, Horn T, Boutros M (2005) E-RNAi: a web application to design optimized RNAi constructs. Nucleic Acids Res 33:W582–W588. doi:10.1093/nar/gki468. Web Server issue

Zha W, Peng X, Chen R, Du B, Zhu L, He G (2011) Knockdown of midgut genes by dsRNA-transgenic plant-mediated RNA interference in the hemipteran insect *Nilaparvata lugens*. PLoS One 6(5):e20504. doi:10.1371/journal.pone.0020504

Zhang H, Li FL, Cheng C, Jiao DX, Zhou Z, Cheng LG (2013) The identification and characterisation of a new deltamethrin resistance-associated gene, UBL40, in the diamondback moth, *Plutella xylostella* (L). Gene 530:51–56. doi:10.1016/j.gene.2013.07.075

Zhang X, Zhang J, Zhu KY (2010) Chitosan/double-stranded RNA nanoparticle-mediated RNA interference to silence chitin synthase genes through larval feeding in the African malaria mosquito (*Anopheles gambiae*). Insect Mol Biol 19:683–693

Zhang X, Mysore K, Flannery E, Michel K, Severson DW, Zhu KY et al (2015) Chitosan/interfering RNA nanoparticle mediated gene silencing in disease vector mosquito larvae. J Vis Exp 97:52523. doi:10.3791/52523

Zhang Y, Wiggins B, Lawrence C, Petrick J, Ivashuta S, Heck G (2012) Analysis of plant derived miRNAs in animal small RNA datasets. BMC Genomics 13:381

Zhao HM, Yi X, Hu Z, Chen SH, Dong XL, Gong L (2013) RNAi-mediated knockdown of catalase causes cell cycle ar rest in SL-1 cells and results in low survival rate of *Spodoptera litura* (Fabricius). PLoS One 8:e59527

Zhao YY, Yang G, Wang-PG YMS (2008) *Phyllotreta striolata* (Coleoptera: Chrysomelidae): arginine kinase cloning and RNAi-based pest control. Eur J Entomol 105:815–822

Zhou XG, Wheeler MM, Oi FM, Scharf ME (2008) RNA interference in the termite *Reticulitermes flavipes* through ingestion of double-stranded RNA. Insect Biochem Mol Biol 38:805–815. doi:10.1016/j.ibmb.2008.05.005

Zhu F, Xu J, Palli R, Ferguson J, Palli SR (2011) Ingested RNA interference for managing the populations of the Colorado potato beetle, *Leptinotarsa decemlineata*. Pest Manag Sci 67:175–182. doi:10.1002/ps.2048

Zhu F, Cui Y, Walsh DB, Lavine LC (2014) Application of RNA interference toward insecticide resistance management. In: Chandrasekar R, Tyagi BK, Gui ZZ, Reeck GR (eds) Short views on insect biochemistry and molecular biology, vol 2. International Book Mission, Academic, Manhattan, pp 595–619. Chapter-27

Zhu J-Q, Liu S, Ma Y, Zhang J-Q, Qi H-S, Wei Z-J et al (2012) Improvement of pest resistance in transgenic tobacco plants expressing dsRNA of an insect-associated gene EcR. PLoS One 7:e38572. doi:10.1371/journal.pone.0038572

11 Egg-Laying Behaviour of *Caryedon serratus* (Olivier) on the Essential Oils of *Skimmia anquetilia*

Manjul Gondwal, Bhanu Pratap Singh Gautam, and Navneet Kishore

Abstract

Nature has been a good source of medicinal agents for thousands of years, and a vast number of modern drugs have been isolated from natural sources based on the information about their uses in traditional medicine. The genus *Skimmia* contains essential oils, coumarins and alkaloids. The principal constituent of essential oil, 'linalyl acetate' is used in manufacture of cosmetics, perfumery and flavouring. Egg-laying behaviour/antifeedant activity of *Caryedon serratus* (Olivier) on the essential oils of flower and leaf of *Skimmia anquetilia* was studied by choice experiment and was observed that number of eggs decreased as the concentration of oil increased. The maximum number of eggs was observed on solvent control. This showed egg-laying deterrent activity in flowers as well as leaves with essential oil of *Skimmia anquetilia* at 1.5% concentrations.

M. Gondwal
Department of Chemistry, Hemwati Nandan Bahuguna Garhwal University, Srinagar 246174, India
e-mail: m.gondwal@gmail.com

B.P.S. Gautam
Department of Chemistry, Banaras Hindu University, Varanasi 221005, India
e-mail: gautambps@gmail.com

N. Kishore (✉)
Department of Plant Science, University of Pretoria, Pretoria 0002, South Africa
e-mail: kishore.navneet6@gmail.com

D. Kumar, C. Gong (eds.), *Trends in Insect Molecular Biology and Biotechnology*, https://doi.org/10.1007/978-3-319-61343-7_11

11.1 Introduction

In many parts of the tropics, mainly in semiarid areas, groundnut (*Arachis hypogaea* L.) that belongs to the family Leguminosae (Fabaceae) (Beghmin et al. 2003) is an important commercial crop, also known as peanut earthnut, goobers, pinders, etc. Other members of this family include cowpea, soybeans, tamarind, melon, etc. (Ashle 1993). After the sixteenth century, Portuguese introduced groundnut into Nigeria although groundnut originated from South America (Brazil) (Adeyemi 1968). Groundnut production in Africa has been estimated at 4.6 metric tons, with Nigeria being the major producers in Africa (Ashle 1993). According to Nyilra, Nigeria's production of unshelled nut is about 2.6 metric tons annually from a land area of approximately 2.5 million hectares (Nyilra 1988). Groundnut thrives best on a well-drained sandy-loam soil; this type of soil facilitates easy penetration of pegs and their development, hence their harvesting (Yayock 1984). Weiss (2000) suggested that temperature range of 25–30 °C, rainfall of 500–1000 mm and a PH range of 6.0–6.5 are considered optimum for groundnut production (Weiss 2000). Groundnut is a major cash crop which serves as a foreign exchange earner prior to the petroleum boom in Nigeria (Adeyemi 1968). According to Aribisala, the crop is a good source of protein, fats and oil, vitamins, etc. Shelled groundnuts are fried, roasted and salted which is eaten as snacks (Aribisala 1993). The crop serves as raw materials for some food industries and also as feed concentrate for livestock (Oaya et al. 2012).

Groundnuts are very important source of nutrition in human diet, are often consumed either directly or as oil and are affected mainly by insects and pathogens. Bruchid beetle, *Caryedon serratus* (Oliver), is one of the major storage pests affecting the groundnut produce causing damage up to 70–80% in stored groundnuts (Harish et al. 2012). It seems that in storage bruchids, besides causing direct damage to groundnut, increase the contamination of aflatoxin in the stored groundnuts. Aflatoxin shows considerable significance due to its deleterious effects on human being, poultry and livestock (Abbas 2005; Chaytor et al. 2011; Diaz et al. 2010; Hifnawy et al. 2004; Iheshiulor et al. 2011; Taranu et al. 2010; Vijayasamundeeswari et al. 2009; Williams et al. 2010). It is a potent carcinogenic, mutagenic and immunosuppressive agent, produced as secondary metabolites by the fungi, *Aspergillus flavus*, *A. parasiticus* and *A. nomius*, on a variety of food commodities (Essono et al. 2009; Kurtzman et al. 1987).

Among the insect pests attacking the stored groundnuts, the groundnut seed beetle (bruchid), *Caryedon serratus* (Olivier), is the only insect species known to infest kernels and intact pods and is thus potentially the most important pest of unshelled groundnut. The bruchid, *Caryedon serratus* (Olivier), is a serious pest of legumes in West Africa, besides other hosts such as *Acacia arabica*, *Tamarindus indica* and *Bauhinia rufescens* (Hall 1954; Diaollo and Huignard 1993). In Sudan, it attacks groundnuts in the Rahad Scheme and also other parts of the country. The literature on the biology of *C. serratus* is scanty and is only available for the other bruchids, *Callosobruchus maculatus*, and other related species, *C. chinensis*. There is much confusion in the past regarding the identity of the groundnut borer, *Caryedon*

serratus. More than 20 names were used before the name *C. serratus* was adopted (Murkerji and Chatterjee 1957). *C. serratus* was given several common names, e.g. tamarind beetle, groundnut bruchid, groundnut seed beetle and groundnut borer (Davey 1958; Southgate and Pope 1957; Green 1960).

Olivier *C. serratus* was described as having a prognathous head type and distinct serrate antennae (Singh 1977). Its body length varied from 6 to 7 mm and has dark reddish brown spots with smudgy black spots on the wings. It has large prominent compound eyes and is distinguished by the presence of broad-hind femur with conspicuous comb of spines. The femur has a strong spike in the middle followed by 10–14 smaller spikes. Davey studied the gender characteristics of *C. serratus* and found that the abdominal segments of the female are wholly covered by the elytron, while that of the male is partly covered, i.e. the elytron doesn't reach the last abdominal segment (Davey 1958b). Sexual dimorphism was distinguished by observing the pygidium. In the case of males, pygidium projected downwards so that in dorsal view it was hidden by the elytra, whereas in females pygidium projected beyond the elytra and dorsally visible. The observations indicated that the female bruchid lived longer than males (Figs. 11.1 and 11.2).



Fig. 11.1 Larva, adult male and female of *C. serratus* (Issoufou et al. 2016)

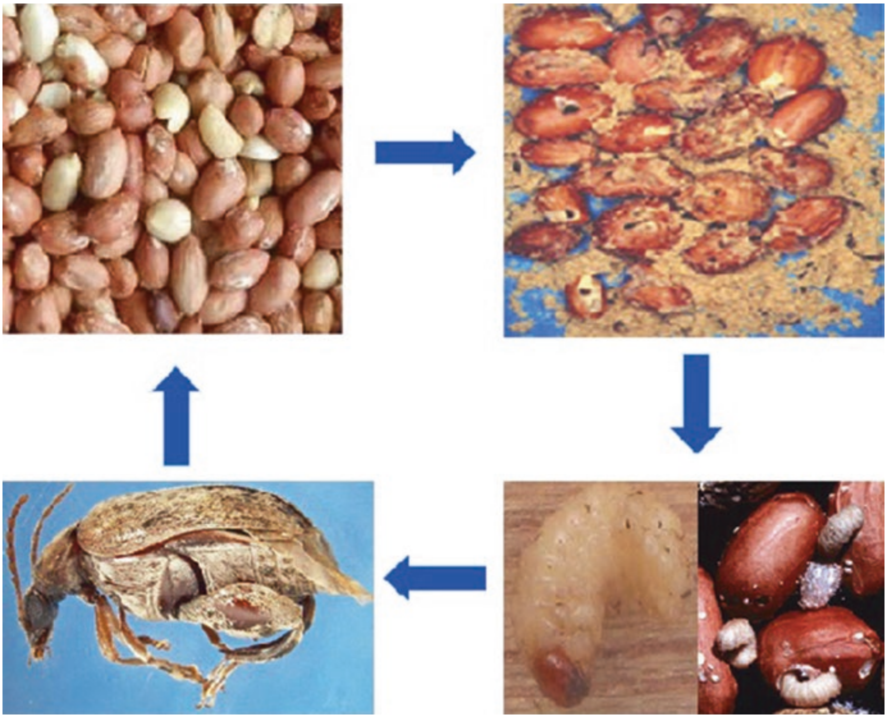


Fig. 11.2 Life cycle and damage of *C. serratus* (Ouedraogo et al. 2016)

11.2 Egg-Laying Behaviour/Antifeedant Activity Against *Caryedon serratus* (Olivier)

Groundnut (*Arachis hypogea* L.) is an important legume cash crop for the tropical farmers and its seeds contain high amount of edible oil (43–55%) and protein (25–28%) (Reddy et al. 2003). Groundnuts are susceptible to the attack of many insect pests when stored. Among these, the groundnut seed beetle (bruchid), *Caryedon serratus* (Olivier), is the only insect species known to infest kernels and intact pods and is thus potentially the most important pest of unshelled groundnut (Devi and Rao 2005).

Antifeedant activity of extracts of *Skimmia anquetelia* and *Aegle marmelos* (Rutaceae family) against the forest pest *Plecoptera reflexa*, *Popular defoliator*, *Clostera reflexa*, *Bamboo leafroller* and *Crysiptya coclesalis* was reported (Negi et al. 2006). Prates reported that some essential oils have acute toxicity, repellency, feeding inhibition or harmful effects on the reproductive systems of insects. Leaf powder, seed kernel powder and oil extracted from the seeds of *Azadirachta indica* and leaf powder and oil extracted from the leaves of *Eucalyptus camaldulensis* and benzene hexachloride (BHC) were tested at 1, 3 and 5% against *Caryedon serratus* (Olivier) (Prates et al. 1998). Eucalyptus leaf oil and neem oil at 3 and 5% were as efficient as BHC and significantly (P = 0.0001) reduced egg laying by *C. serratus*, whereas Eucalyptus leaf powder had no significant effect (Atta and Ahmed 2002).

Some products from plant origin such as neem seed kernel powder, neem leaf powder and *Lantana camara* leaf powder at 25 g/kg groundnuts pods and two aromatic oils (*Citronella* and *Palmarosa*) at 15 mL/kg pods against the groundnut bruchid, *Caryedon serratus*. It was found that *Citronella* oil and *Palmarosa* oil gave total protection to groundnut pods by inhibition of oviposition by the bruchid for 6 months with an efficacy equal to that of malathion dust (malathion 5D). Among the plant powders, *L. camara* had a good oviposition deterrent activity but lost effectiveness gradually after 1 month (Kumari et al. 1998).

11.3 Essential Oils from Medicinal Plants

From thousands of years, nature has been a source of medicinal agents, and a number of modern drugs have been isolated from natural sources based on the information about their use in traditional medicine (Cragg and Newman 2001). The herbal medicines have been derived from rich traditions of ancient civilizations and scientific heritage because the evidences of the use of traditional medicaments in Indian, Chinese, Egyptian, Greek, Roman and Syrian texts date back to about 5000 years (Kamboj 2000). Natural product extracts of therapeutic uses are of immense importance as reservoirs of structural and chemical diversity. A review on national pharmacopoeias from several countries reveals that at least 120 distinct chemical substances from different plants have utility as life-saving drugs (Chauhan and Tiwari 2003). Natural products are obtained from plants, microbes and animals. Among these, plants are more reliable and useful because of easy availability and higher concentration of constituents. Some of the plant products nowadays are used either in their natural form or as derivatives were used originally for other purposes, such as arrow poisons, as part of the religious or other rituals and even as cosmetics (Gupta 1993). Terpenes are among the most widespread diversified and chemically interesting group of natural products that are typically found in higher plants, mosses, liverworts, algae and lichens, although some are of insect or microbial origin (Langenheim 1994). The plants of family Rutaceae consist of 162 genera and 1650 species and are generally shrubs, trees or sometimes herbs, with aromatic volatile oils contained in glands visible at the surface of leaves (Throne 2007). The family Rutaceae is of great economic importance as the source of *citrus* fruits for commerce such as the citrons, lemons, limes, oranges, pomelos (Genus: *Citrus*), the kumquat (*Fortunella*) and the trifoliate orange (*Poncirus*). Rutaceae also includes many ornamental plants such as *Choisya ternata*, *Murraya paniculata* and *Severinia buxifolia* which are cultivated for their glossy green foliage, sweet-scented flowers or bright attractive fruits (Nair and Nayar 1997). In India, the family Rutaceae is represented by 29 genera and 114 species (Karthikeyan 2000). In Uttarakhand, the family Rutaceae is represented by 15 genera and 29 species (Uniyal et al. 2007).

The genus *Skimmia*, belonging to the family Rutaceae, contain about seven or eight species distributed in the Himalayas, East Asia and the Philippines (Gaur 1999). They are generally evergreen shrubs and small trees. The plant is strongly scented and locally used to manufacture incense sticks (Nair and Nayar 1977). *Skimmia* species are used as ornamentals, condiments, food-flavouring agent and

also in the manufacturing of scenting soaps. The leaves are used as insecticides, pesticides and also in the treatment of smallpox, cold, fever and headache (Ahmed et al. 2004; Qureshi et al. 2009). *Skimmia* species show antibacterial, antifungal and antifeedant activity (Sampurna and Nigam 1979; Ahmad and Sultana 2003). The genus *Skimmia* is a rich source of essential oils, coumarins and alkaloids (Wu 1987; Rahman et al. 1998; Razdan et al. 1988). The principal constituent of essential oil, 'linalyl acetate', is very important in the manufacture of soaps, cosmetics, perfumery and flavouring (Skaria et al. 2007). The essential oil is antiseptic and found to be effective against *Staphylococcus* and *Streptococcus* bacteria. Some feed-deterrent and antitumour constituents have also been reported from *Skimmia* species (Escoubas et al. 1992; Hashi 1991). Leaves are used as incense in various religious Hindu rites, eaten in curries by hill tribes and also used for flavouring food in Kashmir (Skaria et al. 2007). In China, it is used for darkening hair and also for hair washing (Skaria et al. 2007). The soot obtained from the burning of leaves is inhaled for the treatment of body pain, fever and flu by the local population of Hazara, Pakistan (Sultana et al. 2002).

Skimmia anquetilia N.P. Taylor & Airy Shaw (Rutaceae) is an aromatic evergreen erect or creeping shrub (up to 1.5 m high) found in the subalpine region of the Garhwal Himalayas (Gaur 1999). There are about 7–8 species of genus *Skimmia* distributed in the Himalayas, East Asia and the Philippines, out of which five species are found in India. It is locally known as Nair patti, Nayalpatti or Nihar in Kumaoun; Kasturchara or Gurlpatta in Jaunsar; Nair in Garhwal, Patar and Nar, near Kashmir; Nar, Barru, Shalangli or Patrang in Punjab; and Kedar patti in various hill regions (Goel et al. 1989). It has the following characteristics: evergreen, usually dioecious or monoclinous, unarmed, leaves alternate, simple, terminal inflorescences, thyrsiform, sepals (3 or) 4 or 5(–7), distinct or basally connate, petals (3 or) 4 or 5(–7), imbricate in bud, stamens (3 or) 4 or 5(–7), distinct, rudimentary in female flowers, with a fleshy drupaceous berry fruit, with 1–5 one-seeded leathery pyrenes and with seeds that are ovoid to ellipsoid. Five to six species are found in the East, South and Southeast Asia (Zhang et al. 2008).

11.4 Egg-Laying Behaviour

Different concentrations of flower oil and leaf oil of *Skimmia anquetilia* were prepared in water containing Tween 20 as surfactant as given below:

1. C_1 (0.5%) = Add 0.5 mL of oil in 99.5 mL of water containing 1 drop of Tween 20.
2. C_2 (1.0%) = Add 1.0 mL of oil in 99.0 mL of water containing 1 drop of Tween 20.
3. C_3 (1.5%) = Add 1.5 mL of oil in 98.5 mL of water containing 1 drop of Tween 20.
4. C (solvent control) = 100 mL of water containing 1 drop of Tween 20.
5. U (control, untreated).

11.5 Source and Maintenance of Culture

The studies on egg-laying behaviour of flower and leaf oil of *Skimmia anquetilia* were carried out with *Caryedon serratus* (Olivier). Nucleus culture of *Caryedon serratus* (Olivier) was obtained from IICT Hyderabad and maintained on groundnuts in the incubator at 35 ± 2 °C and 70% relative humidity at the Department of Chemistry, G. B. Pant University of Agriculture and Technology, Pantnagar.

11.6 Choice Experiment and Observation for Adult Emergence

To investigate the egg-laying behaviour of flower and leaf oil of *Skimmia anquetilia* oil against *Caryedon serratus* (Olivier), open choice experiment was conducted at room temperature (35 ± 2 °C) (Sundria and Kumar 2004). For these experiments, glass petri dishes (diameter 180 mm) were used. On each glass petri dish, the five equidistant circles were marked and labelled as C_1, C_2, C_3, C and U where C_1 stands for concentration (0.5%), C_2 stands for concentration (1.0%), C_3 stands for concentration (1.5%) of oils, C stands for solvent control (water + tween 20) and U stands for untreated groundnuts (Figs. 11.3 and 11.4). Ten unshelled groundnuts were used for each treatment. The unshelled groundnuts were dipped in different concentrations of oils and control and dried at room temperature to evaporate the solvent. Then, the treated and untreated groundnuts were placed in their respective labelled circles. Each treatment was replicated thrice. Freshly immersed five pairs of insects, i.e. five males and five females of *Caryedon serratus* (Olivier), were released in the centre of the petri dish per replication. The petri dish was finally covered and left for mating and egg laying. The number of eggs and number of dead insects were counted after each 24 h till all insects were dead. The groundnuts which contained eggs were removed and replaced with freshly treated groundnuts. The removed groundnuts were collected in separate boxes, respectively, and development of eggs to adults was observed.

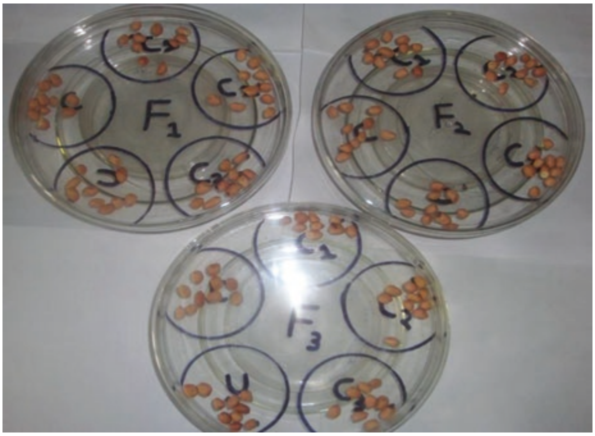


Fig. 11.3 Choice experiment of flower oil of *Skimmia anquetilia*



Fig. 11.4 Choice experiment of leaf oil of *Skimmia anquetilia*

11.7 Fecundity of *C. serratus*

The present studies on egg-laying behaviour of essential oils of flowers and leaves of *Skimmia anquetilia* against *Caryedon serratus* (Olivier) by choice experiment were carried out on three concentrations of oils (0.5, 1.0 and 1.5%), solvent control (water + tween 20) and control (untreated) groundnuts. It was observed that the number of eggs decreased as the concentration of oil increased. The maximum numbers of eggs was observed on solvent control (Tables 11.1 and 11.2).

In flower and leaf oil, almost similar trend for egg-laying response was observed. These results have also been shown in Figs. 11.5 and 11.6.

In flower oil maximum numbers of eggs were observed on solvent control followed by C_1, untreated, C_2 and C_3 groundnuts.

The observations on the number of eggs laid per day in each replication of flower oil have clearly shown that egg laying decreased day by day and the maximum number of eggs was laid in first 6 days and then was almost arrested. Similar trend was also observed in solvent control (water + tween 20) and control (untreated) groundnuts.

In the case of leaf oil, surprisingly the number of eggs laid on the first day was less as compared to the second, third and fourth day. The egg laying was arrested completely on the eighth day. Similar trend was observed in solvent control (water + tween 20) and control (untreated) groundnuts. The results have been showed in Figs. 11.7 and 11.8.

In both the cases, the maximum number of eggs was observed on solvent control and minimum number of eggs on maximum-tested concentration (1.5%) of oil. Generally, female insects prefer egg laying where more food security is available for development of future generation. In this study, it has been clearly shown that higher concentration of oil reduces egg laying. Similar results on neem oil have been reported by Atta and Ahmed.

Table 11.1 No. of eggs laid by *Caryedon serratus* on unshelled groundnuts treated with 0.5 %, 1.0 % & 1.5 % concentrations (C1, C2 & C3) of essential oil from flower treated with control and untreated of *Skimmia anquetilia*.

No of	C_1			Grad		C_2			Grad		C_3			Grad		Control			Grad		Untreated			Grad	
days	F_1	F_2	F_3	total	Mean (±SD)	F_1	F_2	F_3	total	Mean (±SD)	F_1	F_2	F_3	total	Mean (±SD)	F_1	F_2	F_3	total	Mean (±SD)	F_1	F_2	F_3	total	Mean (±SD)
1	8	13	9	30	10.000 ± 2.645	6	16	4	26	8.666 ± 6.429	4	12	6	22	7.333 ± 4.163	10	10	12	32	10.666 ± 1.154	2	7	6	15	5.000 ± 2.645
2	8	11	10	29	9.666 ± 1.527	11	12	5	28	9.333 ± 3.785	5	13	9	27	9.000 ± 4.000	16	13	9	38	12.666 ± 3.511	10	14	9	33	11.000 ± 2.645
3	2	8	12	22	7.333 ± 5.033	2	6	13	21	7.000 ± 5.567	1	4	4	9	3.000 ± 1.732	0	8	9	17	5.666 ± 4.932	7	12	11	30	10.000 ± 2.645
4	5	5	8	18	6.000 ± 1.732	7	3	7	17	5.666 ± 2.309	8	6	7	21	7.000 ± 1.000	4	3	7	14	4.666 ± 2.081	6	7	7	20	6.666 ± 0.577
5	4	6	7	17	5.666 ± 1.527	6	5	6	17	5.666 ± 0.577	2	8	1	11	3.666 ± 3.785	5	5	11	21	7.000 ± 3.461	5	7	3	15	5.000 ± 2.000
6	4	5	1	10	3.333 ± 2.081	4	2	5	11	3.666 ± 1.527	2	4	0	6	2.000 ± 2.000	4	2	1	7	2.333 ± 1.527	2	3	0	5	1.666 ± 1.527
7	2	1	0	3	1.000 ± 1.000	0	0	0	0	0.000 ± 0.000	1	1	1	3	1.000 ± 0.000	3	0	0	3	1.000 ± 1.732	1	0	0	1	0.333 ± 0.577
8	0	0	0	0	0.000 ± 0.000	0	0	0	0	0.000 ± 0.000	0	1	0	1	0.333 ± 0.577	1	0	0	1	0.333 ± 0.577	0	2	0	2	0.666 ± 1.154
9	1	0	0	1	0.333 ± 0.577	0	0	0	0	0.000 ± 0.000	0	2	0	2	0.666 ± 1.154	0	2	0	2	0.666 ± 1.154	0	0	0	0	0.000 ± 0.000
10	0	1	0	1	0.333 ± 0.577	0	0	0	0	0.000 ± 0.000	0	1	0	1	0.333 ± 0.577	0	1	0	1	0.333 ± 0.577	0	0	0	0	0.000 ± 0.000
11	0	1	0	1	0.333 ± 0.577	0	0	0	0	0.000 ± 0.000	0	0	0	0	0.000 ± 0.000	0	0	0	0	0.000 ± 0.000	0	1	0	1	0.333 ± 0.577
12	0	0	0	0	0.333 ± 0.577	0	1	0	1	0.333 ± 0.577	0	0	0	0	0.000 ± 0.000	0	1	0	1	0.333 ± 0.577	0	1	0	1	0.333 ± 0.577
Total	34	51	47	132	44.000 ± 8.888	36	45	41	122	40.333 ± 4.509	23	52	28	103	34.333 ± 15.502	43	45	49	137	45.666 ± 3.055	33	54	36	123	41.000 ± 11.357

Table 11.2 No. of eggs laid by *Caryedon serratus* on unshelled groundnuts treated with 0.5 %, 1.0 % & 1.5 % concentrations (C1, C2 & C3) of essential oil from flower treated with control and untreated of *Skimmia anquetilia*.

No of	C_1			Grad		C_2			Grad		C_3			Grad		Control			Grad		Untreated			Grad	
days	L_1	L_2	L_3	total	Mean (±SD)	L_1	L_2	L_3	total	Mean (±SD)	L_1	L_2	L_3	total	Mean (±SD)	L_1	L_2	L_3	total	Mean (±SD)	L_1	L_2	L_3	total	Mean (±SD)
1	3	1	2	6	2.000 ± 1.000	6	5	1	12	4.000 ± 2.645	6	1	0	7	2.333 ± 3.214	7	5	6	18	6.000 ± 1.000	5	1	0	6	2.000 ± 2.645
2	19	12	11	42	14.000 ± 4.358	10	7	14	31	10.333 ± 3.511	11	4	8	23	7.666 ± 3.511	9	20	10	39	13.000 ± 6.082	9	6	10	25	8.333 ± 2.081
3	12	12	11	35	11.666 ± 0.577	6	10	14	30	10.000 ± 4.000	11	6	11	28	9.333 ± 2.886	11	15	6	32	10.666 ± 4.509	14	5	10	29	9.666 ± 4.509
4	9	6	7	22	7.333 ± 1.527	11	11	11	33	11.000 ± 0.000	12	3	26	26	8.666 ± 4.932	13	10	14	37	12.333 ± 2.081	12	4	8	24	8.000 ± 4.000
5	6	1	0	7	2.333 ± 3.214	5	4	4	13	4.333 ± 0.5777	6	3	12	12	4.000 ± 1.732	8	1	2	11	3.666 ± 3.785	8	3	4	15	5.000 ± 2.645
6	4	4	1	9	3.000 ± 1.732	2	8	0	10	3.333 ± 4.163	3	4	7	7	2.333 ± 2.081	5	2	4	11	3.666 ± 1.527	5	5	1	11	3.666 ± 2.309
7	2	2	0	4	1.333 ± 1.154	0	2	0	2	0.666 ± 1.154	2	1	3	3	1.000 ± 1.000	2	3	0	5	1.666 ± 1.527	0	3	0	3	1.000 ± 1.732
8	1	0	0	1	0.333 ± 0.577	0	0	0	0	0.000 ± 0.000	0	0	0	0	0.000 ± 0.000	0	1	0	1	0.333 ± 0.577	0	1	0	1	0.333 ± 0.577
Total	56	38	32	126	42.000 ± 12.490	40	47	44	131	43.666 ± 3.511	51	22	33	106	35.333 ± 14.640	55	57	42	154	51.333 ± 8.144	53	28	33	114	38.000 ± 13.22

Solvent Control > C_1> Untreated, $C_2 > C_3$
In the case of leaf oil, almost similar trend was observed as given below
Solvent Control > C_2 > Untreated, $C_1 > C_3$

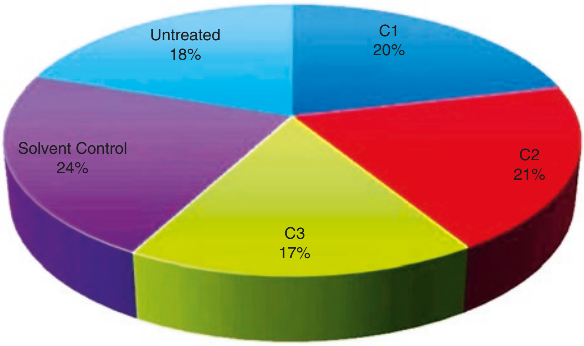


Fig. 11.5 No. of eggs (%) on unshelled groundnuts treated with flower essential oil of *Skimmia anquetilia*

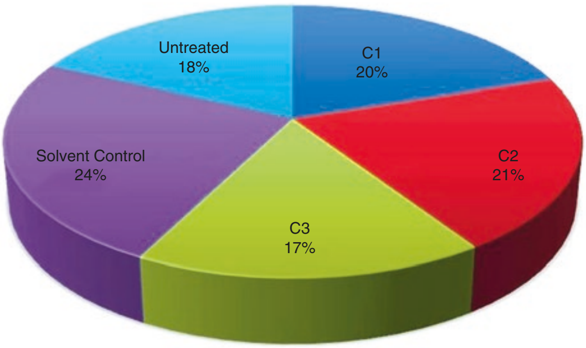


Fig. 11.6 No. of eggs (%) on unshelled groundnuts treated with leaf essential oil of *Skimmia anquetilia*

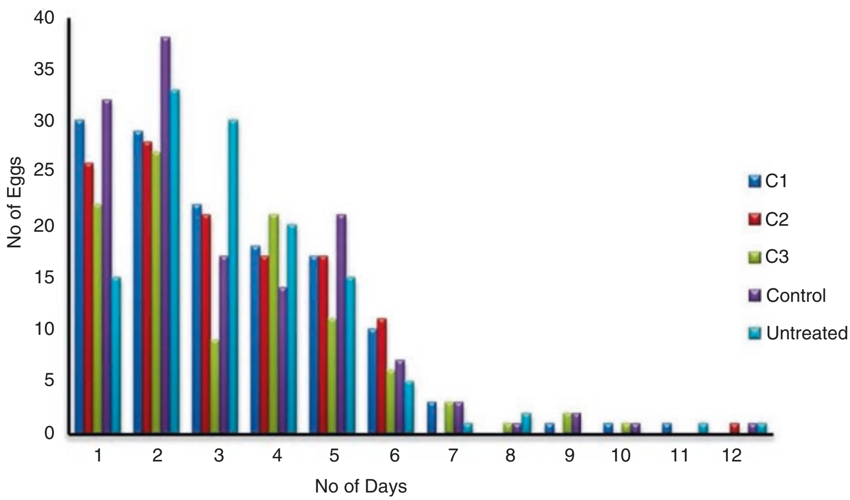


Fig. 11.7 No. of eggs on unshelled groundnuts per day treated with flower essential oil of *Skimmia anquetilia*

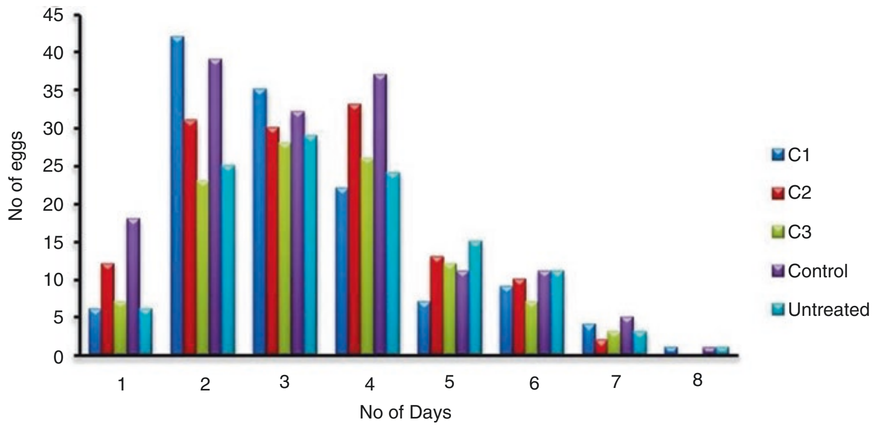


Fig. 11.8 No. of eggs on unshelled groundnuts per day treated with leaf essential oil of *Skimmia anquetilia*

11.8 Molecular Marker Systems in Insects

DNA makers have made a significant contribution to rapid rise of molecular studies of genetic relatedness, phylogeny, population dynamics or gene and genome mapping in insects over the last 15 years (Loxdale and Lushai 1998; Avise 2000; Severson et al. 2001; Heckel 2003). Many improvements have been made to enhance power of resolution (ability to reveal more informative polymorphisms from less number of loci), reproducibility, cost and time consumption in developing and scoring the marker loci. Since then, application of DNA markers in entomology has gone through and is still undergoing a perceptible change in continuously accommodating new technologies for robust and less expensive genotyping methods. The unparalleled advancements in modern molecular biology, particularly in those of DNA marker technology, have already created a wealth of technical know-how that finds useful applications of these markers especially in molecular ecology research in insects (Hoy 2003). Usually, mitochondrial DNA (mtDNA) has been a choice of marker for studying genetic variations in insect species. In addition, mtDNA sequences are often transferred to the nucleus, called nuclear mtDNA (Numt). Variations in copy number and size of Numts are also used to assess the interspecific diversity of these loci in insects (Richly and Leister 2004). Microsatellites are also used as popular markers in insect studies because of high profusion and highly variable nature of their loci in genome. However, the introduction of random amplified polymorphic DNA (RAPD) technique (Williams et al. 1990). The use of PCR (polymerase chain reaction)-based fingerprinting assays gained popularity generally because of the easy-to-perform and easy-to-score procedures for these marker loci. But, because RAPD markers suffer from poor reproducibility, the use of these

markers in insect ecological studies was limited (Black 1993). The advancement of amplified fragment length polymorphism (AFLP) (Vos et al. 1995) technology was adopted as a better substitute to generate more numbers of multiloci reproducible markers, more reliable than RAPD markers. Today, molecular marker technology has reached a new height with the power and the precision of modern genomic tools. High-throughput genotyping methods are now available that can be used for genome-wide mutation screening in hundreds or even thousands of individuals (as much as 300,000 genotypes) as quickly as in a day.

11.9 Conventional Marker Systems

DNA markers such as mtDNA, RAPD, AFLP, microsatellites and ESTs have been used as popular marker systems in insect genetics research. Although there are natural advantages and disadvantages linked with each marker systems, the choice of applying them depends upon the objectives of a study. An additional class of markers generated by arbitrarily primed PCR-based DNA fingerprinting methods, such as RAPD, DNA amplification polymorphisms (DAF) and arbitrarily primed PCR (AP-PCR), are easy to achieve and comparatively easy to score (Black 1993). However, these markers, because of poor reliability and reproducibility, are not appropriate for population studies (Black 1993).

11.10 PCR and DNA Extraction

Sembène et al. (2010) and Vos et al. (1995) extracted, amplified and sequenced DNA with standard protocols described elsewhere. According to them, each sequence was obtained from the DNA of a single seed—beetle. The abdomen, elytra and antennae were kept apart to avoid contamination by fungi and nematodes and to permit consequent morphological observations. A partial cytochrome B (Cyt. B) end region was PCR-amplified using the primers 5′-TATGTACTACCATGAGGACAAATATC-3′ and 5′-ATTACACCTCCTAATTTATTAGGAAT-3′. The ribosomal DNA was targeted for PCR, amplified and sequenced with primer CIL (5′ GCGTTCGAARTGCGATGATCAA 3′) and CIU (5′GTAGGTGAACCTGCAGAAGG3′). For both markers, PCR amplification was performed in 25 μL reaction volume, 2.5 μL enzyme buffer supplied by the manufacturer, 2.5 mM $MgCl_2$, 0.6 unit of Taq polymerase (Promega), 17.5 pM of each primer, 25 nM of each DNTP and 1 μL of DNA extract. They obtained 518 bp of the partial Cyt. B gene in 30 *C. serratus* populations. The arrangement was basic and involved no insertions. The sequences could be clearly aligned and showed 22 different haplotypes due to 51 polymorphic sites. Of these sites, 94% were stinginess informative. The number of nucleotide differences in pairwise comparisons of *C. serratus* populations ranged from 0% to 16.1% due to a large part of *C. serratus* sampled on *C. sieberiana* and the others. Within the same host species, the number ranged from 0% to 0.02%.

Conclusions

In both the essential oils of *S. anquetilia*, the maximum number of eggs was observed in solvent control and minimum number of eggs in maximum-tested concentration (1.5%) of oil. These studies have clearly shown that higher concentration of oil reduced egg laying. Similar results on neem oil have been reported. The essential oils of flowers and leaves of *Skimmia anquetilia* suppressed egg laying by females of *Caryedon serratus*; this effect increases with increase in concentration of oil, but there was no effect on further development of eggs to adults. Essential oils are complex mixture of terpenoids with different quantitative makeup, and due to synergetic effects of constituents, they can be used as safe, eco-friendly and alternative source of synthetic antifeedant and insecticides. Essential oils extracted from *C. schoenanthus*, *L. multiflora* and *O. americanum* have proved lethal concentration (CL50) on adult beetle *C. serratus* thus testifying their efficacy. The egg-laying ability of female *C. serratus* in the presence of essential oils was disturbed and even inhibited. The reduction of females' lifespan equally affected reduction of the number of laid eggs. This inhibition of the egg laying may be attributed to the physiological disturbance caused by essential oils on the females, and in addition, it was reported that monoterpenes inhibit the oviposition of females. Also the egg laying by females of Bruchidae (*C. maculatus* and *C. subinnotatus* PIC) is inhibited by the presence of the essential oil vapours. Essential oil vapours extracted from aromatic plants may cause physiological dysfunction which disturbs the normal functioning of insect ovarioles. This situation may block the sphincters that are likely to push eggs towards the genital opening for their emission. The evaluation of the persistence of essential oils indicated that the effectiveness of oils decreases with the duration of treatment. Similar results were reported on the cowpea beetle *C. maculatus*.

References

Abbas HK (2005) Aflatoxin and food safety. CRC, Boca Raton, FL, p 587

Adeyemi SA (1968) Storage entomology. In: Proceedings of Agricultural Society of Nigeria, vol. 34

Ahmad KF, Sultana N (2003) Studies on bioassay directed antifungal activity of medicinal plants *Calotropis procera*, *Skimmia laureola*, *Peltophorum pterocarpum* and two pure natural compounds ulopterol and 4-methoxy-1-methyl-3-(2'S-hydroxy-3'-ene butyl)-2-quinolone. J Chem Soc Pak 25:328–330

Ahmed E, Arshad M, Ahmad M, Saeed M, Ishaque M (2004) Ethnopharmacological survey of some medicinally important plants of Galliyat areas of NWEP, Pakistan. Asian J Plant Sci 3:410–415

Aribisala OA (1993) Raw material revolution and impact on industrialization in Nigeria. Mednet, Lagos, p 150

Ashle J (1993) Drought and crop adaptation. In: Rowland RJ (ed) Dry land farming in Africa. Macmillan Education, London, p 10

Atta EHA, Ahmed A (2002) Comparative effects of some botanicals for the control of the seed weevil *Caryedon serratus* Olivier (Col., Bruchidae). J Appl Entomol 126:577–582

Avise JC (2000) Phylogeography: the history and formation of species. Harvard University Press, Cambridge, MA, p 684

Beghmin J, Diop N, Sewadah M (2003) The impact of groundnut trade liberalization. Tim Hill Publishing Company Limited India, Mumbai, pp 241–242

Black WC (1993) PCR with arbitrary primers: approach with care. Insect Mol Biol 2:1–6

Chauhan SK, Tiwari G (2003) Effect of sources and levels of nitrogen on partitioning of andrographolide in kalmegh (*Andrographis paniculata* (Burm. F.) Wall. ex Nees.) Indian J Plant Physiol 8:60–62

Chaytor AC, See MT, Hansen JA, de Souza ALP, Middleton TF, Kim SW (2011) Effects of chronic exposure of diets with reduced concentrations of aflatoxin and deoxynivalenol on growth and immune status of pigs. J Anim Sci 89:124–135

Cragg GM, Newman DJ (2001) Medicinals for the milennia. Ann NY Acad Sci 953:3–25

Davey PM (1958) The effect of insect infestation on the quality of decorticated groundnuts with special reference to storage at high and low humidities. Trop Sci 1:296–307

Devi DR, Rao NV (2005) Note on the performance of different groundnut pod protectants against groundnut bruchid, *Caryedon serratus* (Olivier). Legum Res 28:229–230

Diaollo A, Huignard J (1993) Oviposition of four strains of *Caryedon serratus* (Olivier). (Coloeptera: Bruchidae) in the presence of pods or seeds of their wild and cultivated host plants. J Afr Zool 107:113–120

Diaz GJ, Murcia HW, Cepeda SM (2010) Bioactivation of aflatoxin B1 by turkey liver microsomes: responsible cytochrome P450 enzymes. Br Poult Sci 51:828–837

Escoubas P, Fukushi Y, Lajide L, Mizutani J (1992) A new method for fast isolation of antifeedant compounds from complex mixture. J Chem Ecol 18:1819–1832

Essono G, Ayodele M, Akoa A, Foko J, Filtenborg O, Olembo S (2009) Aflatoxin-producing Aspergillus spp. and aflatoxin levels in stored cassava chips as effected by processing practices. Food Control 20:648–654

Gaur RD (1999) Flora of the District Garhwal Northwest Himalaya (with ethnobotanical notes). Trans Media, Srinagar Garhwal

Goel CL, Shiva MP, Mehra SN, Rai YC, Badola KC (1989) Production of essential oil from *S. laureola* (*S. orborescens*) in Uttar Pradesh. Indian Perfum 33:161–164

Green AA (1960) The control of insects infesting groundnuts after harvest in Gambia. Trop Sci 2:130–133

Gupta R (1993) Medicinal and Aromatic plants in India. RAPA Publication, Bangkok, pp 117–180

Hall DW (1954) The quality of the groundnuts from Gambia with special reference to insect infestation. Colon PI Anim Prod 4:227–235

Harish G, Holajjer P, Savaliya SD, Gedia MV (2012) Population density on damage of groundnut by Caryedon serratus. Annu Plant Prot Sci 20:217–219

Hashi M (1991) Antitumour effects and anticomplementary effects of tree polysaccharides. Bulletin of the Forestry and Forest Products Research Institute, Ibaraki, pp 121–148

Heckel DG (2003) Genomics in pure and applied entomology. Annu Rev Entomol 48:235–260

Hifnawy MS, Mangoud AM, Eissa MH, Edin EN, Mostafa Y, Abouel-Magd Y, Sabee EI, Amin I, Ismail A, Morsy TA, Mahrous S, Afefy AF, El-Shorbagy E, El-Sadawy M, Ragab H, Hassan MI, El-Hady G, Saber M (2004) The role of aflatoxin-contaminated food materials and HCV in developing hepatocellular carcinoma in Al-Sharkia Governorate. J Egypt Soc Parasitol 34:479–488

Hoy MA (2003) Insect molecular genetics, 2nd edn. Academic/Elsevier, San Diego, CA

Iheshiulor OOM, Esonu BO, Chuwuka OK, Omede AA, Okoli IC, Ogbuewu IP (2011) Effects of mycotoxins in animal nutrition: a review. Asian Australas J Anim Sci 5:19–33

Issoufou O, Roger NC, Dona D, Wendgoundi G (2016) Insecticide activity of essential oils on the development of eggs and adult of *Caryedon serratus* olivier (Coleoptera: Chrysomelidae), pest of stored groundnut. J Agric Ecol Res Int 9:1–10

Kamboj VP (2000) Herbal medicine. Curr Sci 78:35–39

Karthikeyan S (2000) A statistical analysis of flowering plants of India. In: Singh NP et al (eds) Flora of India, Introductory Vol, Part II. Botanical Survey of India, Calcutta, pp 201–217

Kumari DA, Kumar ST, Reddy VS (1998) Management of groundnut bruchid, *Caryedon serratus* (Olivier) with botanicals in stored groundnut. Pest Manag Econ Zool 6:127–131

Kurtzman CP, Horn BW, Hesseltine CW (1987) Aspergillus nomius, a new aflatoxin-producing species related to Aspergillus flavus and Aspergillus tamarii. Antonie Van Leeuwenhoek 53:147–158

Langenheim JH (1994) Higher plant terpenoids: A phytocentric overview of their ecological roles. J Chem Ecol. 20:1223–1280

Loxdale HD, Lushai G (1998) Molecular markers in entomology. Bull Entomol Res 88:577–600

Murkerji G, Chatterjee S (1957) Morphology of the genital structures of some of the Bruchidae (Lariidae) of Indian and Ceylon and their economic importance. Indian J Entomol 13:1–28

Nair KN, Nayar MP (1977) Rutaceae. In: Hajra PK, Nair VJ, Daniel P (eds) Flora of India. Botanical Survey of India, Calcutta, pp 259–408

Nair KN, Nayar MP (1997) Rutaceae. In: Hajra PK, Nair UJ, Daniel P (eds) Flora of India, vol 4. Botanical Survey of India, Calcutta, pp 259–261

Negi DS, Sharma R, Dashmana PP, Negi P, Mundrawal R, Kalia S (2006) Antifeedant activity of some Rutaceae family plants against forest pests/insects. Acta Cienc Indica 32:209

Nyilra NZ (1988) Pest of grain legumes and their control in Uganda. Academic, London, pp 22–24

Oaya CS, Malgwi AM, Samaila AE (2012) Damage potential and loss caused by the groundnut bruchid caryedon serratus olivier (Coleoptera: Bruchidae) on stored groundnut and tamarind in Yola. IOSR J Agric Vet Sci 1:58–62

Ouedraogo I, Hema SA, Guenda W, Dakouo D (2016) Influence of host plants on the development of *Caryedon serratus* olivier (Coleoptera: Chrysomelidae, Bruchinae), insect pest of groundnut stocks in Burkina Faso. Adv Entomol 4:279–292

Prates HT, Santos JP, Waquil JM, Fabris JD, Oliveria AB, Foster JE (1998) Insecticidal activity of monoterpenes against *Rhyzopertha dominica* (F.) and *Tribolium castneum* (Herbst). J Stored Prod Res 34:243–249

Qureshi RA, Ghufran MA, Gilani SA, Yousaf Z, Abbas G, Batool A (2009) Indigenous medicinal plants used by local women in Southern Himalayan regions of Pakistan. Pak J Bot 41:19–25

Rahman AU, Sultana N, Jahan S, Choudhary MI (1998) Phytochemical Studies on *S. laureola*. Nat Prod Res 12:223–229. http://www.informaworld.com/smpp/title~content=t713398545~db=all~tab=issueslist~branches=12-v1212

Razdan TK, Harkar S, Qadri B, Qurishi MA, Khuroo MA (1988) Lupene derivatives from *S. laureola*. Phytochemistry 27:1890–1892

Reddy TY, Reddy VR, Anbumozhi V (2003) Physiological responses of groundnut (*Arachis hypogaea* L.) to drought stress and its amelioration: a critical view. Plant Growth Regul 41:75–88

Richly E, Leister D (2004) NUMTs in sequenced eukaryotic genomes. Mol Biol Evol 21:1081–1084

Sampurna T, Nigam SS (1979) Antibacterial study of some Indian essential oils. Indian Perfum 23:205–207

Sembène M, Kébé K, Delobel A, Rasplus JY (2010) Phylogenetic information reveals the peculiarity of *Caryedon serratus* (Coleoptera, Chrysomelidae, Bruchinae) feeding on *Cassia sieberiana* DC (Caesalpinioideae). Afr J Biotechnol 9:1470–1480

Severson D, Brown W, Knudson SE, D. L. (2001) Genetic and physical mapping in mosquitoes: molecular approaches. Annu Rev Entomol 46:183–219

Singh T (1977) A key to the northwest Indian bruchids. Ent Mon Mag 113:219–231

Skaria BP (2007) Aromatic Plants, New India Publishing Agency, Pitam Pura, New Delhi, P 210

Southgate BJ, Pope RP (1957) The ground seed beetle. A Study of its identity and taxonomic position. J Nat Hist 10:669–672

Sultana N, Khan MR, Choudhary MI (2002) Triterpene and Coumarins from Skimmia laureola. Natural Product Letters, 16, 305-313

Sundria MM, Kumar A (2004) Biology of groundnuts bruchid, *Caryedon serratus* (OL) on different test hosts. Annu Plant Prot Sci 12:9–12

Taranu IE, Marin DP, Burlacu R, Pinton P, Damian V, Oswald I (2010) Comparative aspects of in vitro proliferation of human and porcine lymphocytes exposed to mycotoxins. Arch Anim Nutr 64:383–393

Throne RF (2007) An Updated Classification of the Class Mangnoliopsida ("Anigospermas"). Bot Rev 73:67–182

Uniyal BP, Sharma JR, Choudhery U, Singh DK (2007) Flowering plants of Uttarakhand (A checklist). Bishen Singh Mahendra Pal Singh, Dehradun, pp 91–92

Vijayasamundeeswari A, Mohankumar M, Karthikeyan M, Vijayanandraj S, Paranidharan V, Velazhahan R (2009) Prevalence of aflatoxin B1 contamination in pre- and post-harvest maize kernels, food products, poultry and livestock feeds in Tamil Nadu, India. J Plant Prot Res 49:221–224

Vos P, Hogers R, Bleeker M et al (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 23:4407–4414

Weiss EA (2000) Oil seed crop. Blackwell, London, p 16

Williams JH, Grubb JA, Davis JW, Wang J, Jolly PE, Ankrah N, Ellis WO, Afriyie-gyawu E, Johnson NM, Robinson AG, Phillips TD (2010) HIV and hepatocellular and esophageal carcinomas related to consumption of mycotoxin-prone foods in sub-Saharan Africa. Am J Clin Nutr 92:154–160

Williams JGK, Kubelik AR, Livak KJ et al (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 18:6531–6535

Wu TS (1987) Alkaloids and coumarins of *S. reevesiana*. Phytochemistry 26:873–875

Yayock JY (1984) A review of the agronomic principle of groundnut production. In: National seminar on groundnut production, Institute of Agricultural Research, Ahmadu Bello University, Zaria, 45 pp

Zhang D, Hartley TG, Mabberley DJ (2008) Flora of China. Science, Beijing, pp 51–98

Part III

Functions and Applications of Insect Derived Products

Research Advancement of Insect Origin Fungus *Cordyceps*

12

Zhungua Pan

Abstract

Chinese caterpillar fungus, *Cordyceps sinensis*, is a traditional Chinese medicine that parasitize Hepialidae larvae and grows on mountain at an altitude of 3000 m high. After infection, the larvae become rigid, latent feel the humidity in the *Cordyceps* topsoil depth of 10 cm, which is formed by a stiff end pumping out insects long rod-like stroma (i.e., *Cordyceps sinensis* fruiting and sclerotium of dead insects (larvae corpse) to form a composite) when the spring comes and snow melt. It is mainly produced in Qinghai, Tibet, Sichuan, Yunnan, Gansu, Guizhou, and other provinces and autonomous regions of the alpine zone and snow-capped mountains and plains.

12.1 *Cordyceps sinensis*

Chinese caterpillar fungus, *Cordyceps sinensis*, is a traditional Chinese medicine that parasitize Hepialidae larvae and grows on mountain at an altitude of 3000 m high. After infection, the larvae become rigid, latent feel the humidity in the *Cordyceps* topsoil depth of 10 cm, which is formed by a stiff end pumping out insects long rod-like stroma (i.e., *Cordyceps sinensis* fruiting and sclerotium of dead insects (larvae corpse) to form a composite) when the spring comes and snow melt. It is mainly produced in Qinghai, Tibet, Sichuan, Yunnan, Gansu, Guizhou, and other provinces and autonomous regions of the alpine zone and snow-capped mountains and plains.

The distribution area of wild *Cordyceps* is narrow, its natural parasitic rate is low, and its living environment is demanding, with limited resources. In recent years, due to serious destruction of the ecological environment of *Cordyceps*, resulting in a reduction of a large number of unreasonably excavated resources, the yield

Z. Pan
School of Biology and Basic Medical Science, Soochow University, Suzhou 215123, China
e-mail: pzh1971@163.com

D. Kumar, C. Gong (eds.), *Trends in Insect Molecular Biology and Biotechnology*, https://doi.org/10.1007/978-3-319-61343-7_12



Fig. 12.1 Morphology of *Cordyceps sinensis*. Being Composition by fruiting body with black color and insect body with yellow color

decreased year by year. Because of the demand of *Cordyceps* as a medicine and food and people's continued pursuit of its new pharmacological effects, many countries have doubled its production, with the prices increasing yearly and the international market becoming increasingly scarce. In order to meet the needs of the global market, researches of *Cordyceps sinensis* have been conducted on some parts of present-day China (Fig. 12.1).

12.1.1 Strain Research

12.1.1.1 Species of Strains

At present, the reports in domestic and related species of *Cordyceps sinensis* strain belonging to ten genera of 30 kinds, such as *Cordyceps* (*Hirsutella hepiti*), hairy spore Chinese bending strength (*Tolypocladium sinensis*), Chinese enzyme (*Chrysosporium sinensis*) from Kim, in comparison, located in Sichuan, Qinghai, Tibet, Gansu, and other main producing areas of *Cordyceps sinensis* were sexual stage for the same species, the asexual stage characteristics are the same as Chinese *hirsutella*. Zhang Zhi and other domestic statistics reported *Hyphomycetes* and *Cordyceps* about as many as 10 genera and 16 species; Shen Nanying (1983) first isolated a *Hyphomycetes* from fruiting bodies and in the solid medium multiple formation of stroma, the morphology, and the natural state under the stroma of approximately the bacteria. Is considered to be a kind of cefotaxine (*Cephalosporium* sp.); Chen Qingtao (1984) from the Kangding mining *Cordyceps* from Sichuan through the organization of isolated strains named China *Paecilomyces* (*Paecilomyces sinensis*, Chen, Xiao & Shi) in 1986 from Sichuan by Hanchuan mining *Cordyceps* from isolated strains named bat moth species mildew (*Mortierella hepiali* Chen & Liu); Wang Qin (1987) isolated Chinese *Cephalosporium* (*Cephalosporium sinensis* Chen; Li Zhaolan (1988) from the Qinghai Longhua area of *Cordyceps sinensis* samples. Isolated bat moth (*Scytalidium hepiali* C.L. Li) column was found, and the effective components of *Cordyceps* and close, the same year, Li Zhaolan also reported

that isolated from Yunnan by Chinese bent neck Diqing (*Tolypocladium sinensis* C.L. Li) on *Cordyceps sinensis*, its chemical composition and natural *Cordyceps* is very similar; Liu Xijin (1989) from Sichuan Kangding production of *Cordyceps* stroma and sclerotium, isolated strains by multiple batches, large quantity, in many ways, China of colony and sporulation characteristics, and reported by Shen Nanying et al. are basically the same, they will be the strain named Chinese *Hirsutella* (*Hirsutella sinensis*, Liu, Guo, Yu & Zeng; Dai Ruqin (1989) from Yunnan, Qinghai, Diqing) Longhua strains named *Paecilomyces hepiali* (*Paecilomyces hepiali*, Chen & Dai Liang Zongqi (1991)) from Lixian of Sichuan *Cordyceps* in sclerotium isolated from another *Hyphomycetes*, named China *Chrysosporium* (*Chrysosporium sinensis* Liang); Wang Wei (1997) isolated from Yunnan Lijiang *Cordyceps* to a new species, named China Verticil (*Verticillium sinensis* Wang sp. nov).

12.1.1.2 Genome Research

The first Chen in 1997, Y of *Cordyceps sinensis* of different kinds and sources are analyzed at the DNA level difference on using RAPD technology, obtained some preliminary judgment; Zhang, reported by Y in 2009 Ophio *Cordyceps sinensis* C018 http://www.ncbi.nlm.nih.gov genome sequence (Gi08351), opened a prelude from the gene level of *Cordyceps sinensis* the study indicated that Ophio *Cordyceps sinensis* gene size 78.52 Mb, 6972 Kuo, H. gene; C. is equal to 2005 and Yang. J in 2008 to collect different types of *Cordyceps* fungus ITS analysis, all strains with the same rDNA-ITS region; Liang, Hong-Hui and other genetic diversity in 2008 of 18 *Cordyceps* species the inter simple sequence repeat (ISSR) markers to assess genetic diversity found in *Cordyceps sinensis*, genetic. The geographic distance and packet mode and latitude gradient matching; Feng Hui (2008) to construct the *Cordyceps sinensis* cDNA library, obtained 6434 sequences by homologous sequence alignment, the prediction of a large number of functional genes of *Cordyceps sinensis*, including a variety of resistance genes and life-related genes.

So far, in the NCBI database, there are about 12813 Chinese caterpillar fungus gene, and its polymorphism was reported, which lay a foundation for further study of *Cordyceps sinensis*.

12.1.2 Functions

12.1.2.1 Immune Functions

Cordyceps sinensis (HW) is commonly used to test cells and mice. *Cordyceps sinensis* is a nonspecific immune enhancer. K. Oh and other hot-water extracts (HW), which were fed to mice by oral feeding, were observed to investigate their effects on the activation of macrophage and intestinal immune system. In vitro test, the supernatant of D mouse Peyer's C3H/Hej cells treated with g/kg/1/d/1 HW1.0 was found in the supernatant of s-patch cells, and the number of bone marrow cells increased to 1.9 times. Under light microscope, compared with the control group, various types of bone marrow cells including macrophages and myeloid cells increased significantly, indicates that the induction of hematopoietic growth factor HW is strong. In addition, it is speculated that there must be some cytokines to mediate this proliferation

reaction, and the presence of GMF and IL-6 was confirmed in the supernatant. The above results showed that the expression of mice orally fed with HW can activate macrophages to regulate the IL-6, at the same time improve the hematopoietic growth factors (such as Peyer's patch cells secreted GM-CSF and IL-6) expression, the latter effect on the immune system, which play the regulating role of the immune system.

Chen et al. reported that the use of PKA and PKC inhibitors in MA-10 mice Leydig tumor cells to do the prognosis, the amount of steroids to reduce the amount of 61%. Moreover, the production of acute steroid production-induced protein (StAR) was induced by the dose and concentration of *Cordyceps sinensis*. The expression was inhibited by PKA and PKC inhibitors. It was showed that the cells stimulated by the activation of PKA and PKC signal transduction pathway stimulated the production of steroids, which mediated immune responses. Kon based on the mycelium of HW is on the antifatigue and anti-stress effect were found in mice fed with 150 and 300 mg-kg-1-d-1 HW after the swimming endurance was significantly prolonged by 75–90 min and with relieve fatigue. When the mice were fed with the 150 mg-kg-1-d-1 8 h, the stress of the adrenal gland, spleen, thymus, and thyroid gland were inhibited when the 48 h was in a state of stress. HW as the brake parameters were also significantly inhibited the increase of total cholesterol and decreased the level of alkaline phosphatase. These effects were related to the improvement of immune function. Studies have reported that *Cordyceps* aqueous extract could significantly inhibit mice spleen cells to concanavalin A (ConA), LPS proliferation, reduce the delayed hypersensitivity induced by allogenic antigen.

12.1.2.2 Hepatoprotective

Protective effects of CC14 and (TAA) on the prevention and treatment of CS in mice with liver injury. Results show that CC14- or TAA-induced liver injury in mice after oral administration of CS liposome, all of which can cause liver damage of ALT have different degrees of decline and the decline range and dose related. Pathological sections of liver tissue in mice showed that there was no significant improvement in the liver tissue of CC14 damage induced by CS liposome, which may be due to the irreversible liver injury induced by TAA in mice.

12.1.2.3 Prevention and Treatment of Hepatic Fibrosis

The therapeutic effect of CS on hepatic fibrosis induced by CC14 was observed in the rats with aqueous solution. Results show that the serum CS of PC was significantly lower than the control group; liver cell degeneration, necrosis, hyperplasia of fibrous tissue was significantly lighter than the control group; CS group I, III, IV collagen deposition in liver tissue is also lighter than the control group and can inhibit the synthesis of collagen. Ultrastructural study showed that CS not only inhibited the proliferation of FSC (in the narrow gap of hepatic sinusoidal cells) but also inhibited the transformation of FSC to fibroblast and fibroblast cells. Therefore, it is speculated that the mechanism of antihepatic fibrosis of CS may be through inhibiting the proliferation of FSC and the transformation of fibroblasts and fibroblasts, thus weakening the ability of FSC to synthesize collagen.

12.1.2.4 Antitumor

Cordyceps Polysaccharide and Water Extract on Meat Bacteria (S180)

Cordyceps polysaccharide and water extract on meat bacteria (S180), Lewis lung cancer, breast cancer, laryngeal cancer (MA-737), and in vitro culture of tumor cells showed a significant inhibitory effect. *Cordyceps sinensis* water extract on mice transplanted Lewis lung cancer primary tumor and spontaneous lung metastasis was significantly inhibited; tumor suppressor rate is 67%. Combined application of *Cordyceps sinensis* mycelium and cancer drug can enhance 6-mercapto purine, cyclophosphamide, vincristine anticancer activity.

The Anticancer mechanism of _Cordyceps sinensis_

The research of anticancer mechanism of *Cordyceps sinensis* is rare, the main effective components of *Cordyceps* 3-deoxyriboside, containing a free alcohol-based incorporation of cancer cell function in DNA, and inhibition of nucleoside or nucleotide phosphate to nucleic acid synthesis to generate two phosphate and three phosphate derivatives to inhibit cancer cells; a large number of D-mannitol sugar and polysaccharides are a regulator, and nonspecific immune enhancement can activate the body's immune cells, especially T lymphocytes and monocytes-macrophage system poly-lymphokines, and attack cancer cells, exerting its antitumor effect.

Study on the Apoptosis of Tumor Cells

Study on the apoptosis of tumor cells induced by Lee found that the extract of *Cordyceps sinensis* mycelium can inhibit human myeloid leukemia cell growth and induce its apoptosis, *Cordyceps sinensis* hot-water extract showed toxic effects on HL-60 cells, and the IC50 value is 0.8 mg-mL-1, the fracture in a time- and concentration-dependent manner in HL-60 cells induced by DNA. In the process of apoptosis, Caspase3 and specific proteolytic cleavage of poly (PARP) were detected, the results show that *Cordyceps sinensis* HW through activation of Caspase3 induced apoptosis to inhibit the proliferation of tumor cells. Therefore, it has the potential for the treatment of human leukemia. Some scholars of B16 melanoma cells in mice by tail vein injection (C57BL/6) the first day, every 2 days on the fed with different doses of artificial cultivation of *Cordyceps* polysaccharide 27D table, lung tissue immunohistochemical study found that compared with the control group, the c-my table *Cordyceps* polysaccharide processing group, expression c-Fos and VEGF decreased significantly ($P < 0.05$). It showed that the polysaccharide from *Cordyceps sinensis* could inhibit the growth of tumor in mice liver and lung tissue, which may be the adjuvant of tumor therapy.

12.1.3 Study on Active Components

Two kinds of effective components of *Cordyceps* currently recognized as cordycepin and *Cordyceps* polysaccharide, adenosine, mannitols, etc.

12.1.3.1 Cordycepin

Cordycepin is 3′-deoxy adenosine or deoxyadenosine (3-deoxyadenosine), nucleic acid derivative containing nitrogen glycosides, is a purine alkaloid, and is a kind of nucleoside antibiotics. It is an important active component of *Cordyceps sinensis*, which has significant pharmacological effects. As early as 1970s in the twentieth century, it was found that the effect of the inhibition of tumor, anti-plasmodium, and mRNA translation was found to have a role in the inhibition of tumor. A 1990s study found that plays an important role in adding the expression of adenosine deaminase inhibitor on the antitumor activity of cordycepin, to achieve a breakthrough, the United States will start in 1997 for a period of cordycepin in clinical trials, treatment of acute anterior B and T lymphocytic leukemia patients, at the same time, cordycepin also exhibit antifungal strong, anti HIV-virus type, and selective inhibition of clostridium bacteria activity. The synthesis of cordycepin can interfere with RNA and DNA cells, inhibit abnormal cells (cancer cells) of the division and different as the difference in cell RNA polymerase tool, and has the special effect to protect life and repair gene, genetic code, now cordycepin in the United States as anticancer, antiviral drugs has entered the three phases of beds.

At present, the domestic and foreign reports that the artificial synthesis of Chinese caterpillar fungus can also be extracted from the Chinese caterpillar fungus, but the yield is very low, only a small amount of the product supply of Chinese caterpillar fungus, the market price is 2 070 000 dollars/kg (more than 98%). It can be expected that with the application of *Cordyceps* in the field of medicine and the development of related products, market demand will be greatly increased, so the market potential is huge, its extraction and purification methods of research are worth the attention.

12.1.3.2 *Cordyceps* Polysaccharide

According to pharmacology and clinical research, *Cordyceps sinensis* polysaccharide can improve immunity, antitumor agents, and so on, so the research of *Cordyceps* polysaccharide is a hot issue in biological research. For the study of fungal polysaccharides in the United States, Japan, Germany, Russia, and other countries in a leading place, China is still in its infancy. *Cordyceps* worm is hydrolyzed by hydrochloric acid, NaOH, and protein, ethanol precipitation, standard Fehling reagent titration, the measured raw sugar is 23.87%. Anthrone sulfuric acid colorimetric method for the determination of the sugar in the 42.0% [28] by Bai Yune. Yuan Jianguo through the purification and breeding of strains, a soluble *Cordyceps* polysaccharide content is the highest, so the original *Cordyceps* polysaccharide content for mycelial dry weight increased from 6–7% to 10–12%; and the polysaccharide component analysis showed that the polysaccharide is composed of mannose, galactose, glucose, and other monosaccharide composition. Galactomannan, by D-galactose and mannose D-composition according to Moore, the branch with the height of the structure, the main chain (1–2) were mannose based exist, also has its branches (1–5) galactofuranose.

A polysaccharide CS-F10 was isolated from the mycelium of artificial medium. It is composed of galactose, glucose, and mannose in a molar ratio of 43:33:24, and

the molecular weight is about 15,000. Shen Min reported by the gel filtration method to measure *Cordyceps* polysaccharide molecular weight of 43,000, was composed of mannose, galactose, and glucose =10.3:3.6:1. Su Pu in Tibetan Medicine Research reported in the separation of two kinds of polysaccharides from *Cordyceps sinensis*, a molecular weight of approximately 23,000. The monosaccharide composition of D-mannitol and D-galactose, the molar ratio of 3:5; another and Shen Min reported the same. Yuan Jianguo and other kinds of polysaccharides were separated into seven groups, the molecular weight of these seven kinds of polysaccharides and the composition and molar ratio of the sugars were different. Sasaki et al. confirmed that the antitumor activity of the fungal polysaccharide was related to the molecular weight, only when the molecular weight is more than 16,000; it has the antitumor activity.

12.1.4 Origin and Distribution

Cordyceps sinensis grows in the mountains at 3000–5000 m above sea level. From a worldwide perspective, *Cordyceps* is only distributed in four countries such as China, Nepal, Bhutan, and India. (Fig. 12.2)

In China, the main *Cordyceps* bat moth larvae parasitized in Qinghai Tibet Plateau alpine environment, mainly distributed in Qinghai, Yunnan, Sichuan, Tibet, and Gansu provinces, including Qinghai *Cordyceps* best quality, maximum yield. According to records, in the 1980s the annual production of *Cordyceps sinensis* in Qinghai province is 30~35t, accounting for about 70~80% of the national



Fig. 12.2 The environment of *Cordyceps sinensis* growth. The *Cordyceps sinensis* is the worm being infected by *Cordyceps sinensis* and the fruiting body being grow out of the ground when the snow to melt causes by more and more warmth weather on mountain

production, the main producing areas of Yushu and Golog, two states, especially in the highest yield of Yushu *Cordyceps sinensis*.

12.1.5 Advances in Artificial Cultivation

12.1.5.1 Study on Strain

Artificial cultivation of *Cordyceps sinensis* strains has been resolved, at present, a large number of studies show that the strain research has been mature. Stensrud, O in 2007, by ITS analysis that *Cordyceps sinensis* is a parasitic fungus compound, and more and more scholars believe that *Cordyceps sinensis* is a single bacterium, only to adapt to the environment of gene mutations. In recent years, with the production process of silkworm, *Cordyceps* research on *Cordyceps sinensis* strain induced mutation, mutant screening suitable for infection of silkworm, and has made some progress.

12.1.5.2 Host Studies

In 2003, Chen et al. reported on the biology of *Cordyceps sinensis*, such as the moth eggs. In 2006, Liu et al. reported on the species and distribution of insects in *Cordyceps sinensis*. Zhang et al. (2009) reported the study of captive larvae, Wang and X.L. were equivalent to the host species of *Cordyceps sinensis* in 2011. It is expected to find suitable hosts that can be easily bred and cultivated and found that 13 species of 91 species of insect hosts can infect *Cordyceps sinensis*. In recent years, a large number of reports on the host of *Cordyceps sinensis* have been able to artificially reared bat moth larvae, a typical 2002 Wang Hongsheng and other research. If seed production can be solved, *Cordyceps sinensis* will be fully capable of artificial production.

12.1.6 Development of Product Research

Cordyceps resources are increasingly scarce, therefore, most of the current use of *Cordyceps sinensis* fungus fermentation of hyphae.

12.1.6.1 *Cordyceps sinensis* Cephalosporium

From the *Cordyceps sinensis* production in Qinghai, is a semi-known fungi, Congmuispora, Cladosporangia, Cephalosporium family, Cephalosporium. By the Qinghai Institute of Animal Husbandry and Veterinary Medicine and Hangzhou, the second pharmaceutical factory made “Ning Xin Bao” capsule for the treatment of patients with bradyarrhythmias, can improve the sinus node and atrioventricular conduction function and improve sinus rhythm. With anti-inflammatory, immune, and the role of hepatitis treatment, while improving sleep and appetite.

12.1.6.2 *Paecilomyces* Bat Moth

From Hualong County of Qinghai, *Cordyceps* produced on the isolated, is a semi-known fungi, Cong stems spores, from Stemporaceae, *Aspergillus* family, *Penicillium*.

By the Chinese Academy of Medical Sciences Institute of Medicine and Jiangxi State Pharmaceutical Company made "Jinshuibao" capsule, used in the clinical treatment of chronic bronchitis, hyperlipidemia, and impotence, premature ejaculation, irregular menstruation, sexual dysfunction and other diseases. TCM is a lung, kidney deficiency, kidney yang, the effect is significant and so on.

12.1.6.3 Bat Moth Mortierella

From Sichuan Wenchuan Cordyceps produced on the separation, are algae-like, Mucor, Mesemeriaceae, Mortierella. By the Shanxi Datong Liqun Pharmaceutical Factory and Shanxi Medical Research Institute made "Zhi-Ling" capsule, in the clinical treatment of bronchitis, asthma is better. Can make chronic renal failure, chronic nephritis, anemia in patients with hemoglobin and red blood cells increased significantly on cirrhosis of the liver have a certain effect.

12.1.6.4 Chinese Paecilomyces

Isolated from Sichuan Cordyceps produced *Cordyceps sinensis* isolated, is a semi-known fungus, Congmeposporidium, plexus stem Division, Mortierella is. From Hongshan Pharmaceutical Factory of Fuzhou, the production of "*Cordyceps sinensis*" capsules was carried out by using the surface culture of Paecilomyces chinensis CN80-2 for clinical treatment of hypothyroidism, angina pectoris, allergic rhinitis, conscious tinnitus, chronic bronchitis and Tumor adjuvant therapy.

12.1.6.5 Cylinders

Cordyceps produced from Qinghai, isolated by the Navy Medical Research Institute and the Shanghai Cooperation with the Third Factory made of "Cordyceps" capsule, attending primary thrombocytopenia and chronic obstructive pulmonary disease. In addition, Baoding pharmaceutical plant made of Cordyceps "liver Ganbao" capsule, the treatment of chronic hepatitis; Kunming Kangfu pharmaceutical factory trial production of "*Cordyceps sinensis*" capsules, have a role in arrhythmia. In addition, there are treatment of liver disease, "off Austria Ling" in Cordyceps and so on. In recent years, with the rapid development of nutrition and health products, Cordyceps food series of development has attracted wide attention, the development of varieties is increasing. Its medicinal value is more and more people are happy.

12.1.7 Outcome and Anticipation

We study the ultimate goal of *Cordyceps sinensis* is to use them for the benefit of mankind. One of the foster entity is one of the goals that people pursue. Cordyceps cultivation of artificial research has been nearly 40 years, and now it is difficult to cultivate our laboratory fruiting body (whether artificial or insects on the basis of the body), *Cordyceps sinensis* appears to be large-scale artificial cultivation to achieve There is a long way to go. However, with the completion of the genome sequencing, joint research in different areas of scientific research and attention from all sectors of society, we look forward to large-scale cultivation of *Cordyceps sinensis* bacteria can be achieved as soon as possible.

Cordyceps sinensis as a significant role in health and disease prevention, by the favor of consumers, has broad market prospects. Cordyceps prices are increasing year by year. However, the wild Cordyceps resources atrophy of the situation is increasingly serious, through a variety of ways to ensure their survival and reproduction is a priority. In addition, the current large-scale fermentation of higher fungi production is the current access to drugs and fungi mycelium and metabolic products of the main ways.

In general, *Cordyceps sinensis* health care and efficacy of ingredients has been basically clear, pharmacological effects are becoming increasingly clear, with the social and economic development, people's living standards have been significantly improved, awareness of disease prevention and health care gradually deepened. *Cordyceps sinensis* as a functional ingredient clear, natural non-toxic medicinal, health supplies, will also be more and more consumer groups of all ages.

12.2 *Cordyceps militaris*

Cordyceps militaris (L.) Link, also known as *Cordyceps militaris* (*Cordyceps militaris* (L.) Link), is also known as *Cordyceps militaris* (North Cordyceps), Ascensycota belonging to the fungus community, Hypocreales, clavicipitaceae, Widely distributed species, all provinces and autonomous regions are distributed, the child seat in the spring to fall in semi-buried in the forest or under the foliage of Lepidoptera insect pupae grow.

Cordyceps as a traditional precious traditional Chinese medicine, our people have long been aware of its medicinal value. Li Shizhen (1518) in the "Compendium of Materia Medica," pointed out that the cicada can attending "pediatric Tianjian epilepsy, cry at night, palpitations." According to King of soldiers (1986) research, when the "cicadas" includes the cicada Cordyceps (C.sobolifera) and *Cordyceps militaris* and other parasitic on the body or the pupa body of Cordyceps. *Cordyceps militaris* cultivation since the success of pharmacological and toxicological aspects of research has been widely carried out. Studies have shown that *Cordyceps militaris* and *Cordyceps sinensis* has a very similar role, non-toxic side effects, *Cordyceps sinensis* can be used as a substitute (*Cordyceps sinensis* sace). In recent years, with the people of *Cordyceps militaris* health care efficacy and a variety of medicinal value awareness, its development and utilization of research attention, and in genomics, pharmacology, active ingredients and product development and other aspects of a great deal progress.

12.2.1 Morphological Characteristics

Cordyceps militaris is *Cordyceps militaris* infected silkworm pupae, then pupa was hardening, the appropriate temperature and humidity in the fruiting bodies formed under the growth of insect compound (Fig. 12.2).

12.2.2 Strain Research

The results showed that the genome of *Cordyceps militaris* was 32.2 Mb smaller than that of Metarhizium anisopliae and found that more than 5000 expressed sequence tags were predicted to encode 9684 Protein gene, gene number: AEVU00000000; interproScan analysis identified 2736 conserved proteins. Successful determination of the genome of *Cordyceps militaris* has brought great value to the study of *Cordyceps militaris* (Fig. 12.3).

In the study of functional genes, Zheng Z carried out the transgenic research of *Cordyceps sinensis* in 2011, and concluded that Agrobacterium can be used as a tool to transgene into *Cordyceps militaris*. In 2012, Zheng and Z cloned and analyzed the cDNA of cytochrome C oxidase gene of *Cordyceps militaris* (ORF) encodes a 530-amino-acid protein. In 2012, Zhou XW et al. Cloned and expressed the SOD gene of *Cordyceps sinensis*. Xiong, C. In 2013, It was found that the activity of glutathione peroxidase (GPX) directly affected the activity of *Cordyceps militaris*.

In the classification of bacteria, no systematic research on *Cordyceps militaris* has been found. The main difficulty is that the strains with different morphologies and traits are found, and the resource collection and preservation are difficult and the coefficient of variation is too large.

12.2.3 Effect

12.2.3.1 Anti-Tumor Effect

Cordyceps militaris on a variety of tumors have a good effect. Cho, H. J et al. (2007) studied the effect of cordycepin on tumor promoters, suggesting that cordycepin can inhibit thapsigargin (a tumor promoter); Jeong, M. H in 2014 through the study, the different content of cordycepin *Cordyceps militaris* on tumor-bearing mice model and cell model experiments, confirmed the cordycepin response to CD8 + T cells



Fig. 12.3 Morphology of *Cordyceps militaris*. Being Composition by fruiting body with orange color and brown pupa

have a direct effect, indicating the role of cordycepin anti-tumor immunity mechanism. Cordycepin enrichment of *Cordyceps militaris* is a promising candidate in cancer immune adjuvant;

12.2.3.2 Anti-Microbial

Cordyceps militaris on a variety of pathogenic microorganisms were inhibited. Cordyceps has been found active ingredient in the antibacterial activity of the main component of cordycepin. Ahn, YJ reported in 2000, ten micrograms of cordycepin dose can inhibit Clostridium paraputrificum and Clostridium perfringens, Bifidobacterium breve, Bifidobacterium longum, Bifidobacterium adolescentis, Lactobacillus acidophilus and Lactobacillus casei and other common pathogens.

12.2.3.3 To Protect the Liver and Kidney and Respiratory System

Cordyceps militaris can significantly improve the physical condition of patients with chronic renal failure such as increased creatinine clearance rate, and promote protein synthesis, to correct the negative nitrogen balance, improve the quality of life of patients. Choi, H. N. and other fatty liver using the mouse model of *Cordyceps sinensis* (*Cordyceps sinensis*) on the protection of fatty liver. 4-week-old male mice were fed with AIN-93G diet or 1% *Cordyceps militaris* water extract 1 week after the adaptation of the diet. Serum glucose, insulin, free fatty acid (FFA), alanine aminotransferase (ALT), and proinflammatory cytokines. Lipids, glutathione (GSH) and lipid peroxidation in liver were measured. Results: Cordyceps consumption significantly reduced serum glucose, and the homeostasis model assessed the insulin resistance index (HOMA-IR). In addition to lowering serum levels of FFA, *Cordyceps militaris* significantly decreased total liver lipid and triglyceride levels. Serum ALT activity, tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) levels were reduced by *Cordyceps militaris*. The consumption of *Cordyceps militaris* increased liver GSH and decreased lipid peroxidation levels. Conclusion: These results suggest that *Cordyceps militaris* can play a protective role in the development of anti-fatty liver, on the one hand by reducing inflammatory cytokines and improving the antioxidant status of mouse liver;

12.2.3.4 Eliminate Free Radicals in the Human Body

Cordyceps militaris on the free radicals have a good ability to clear. *Cordyceps militaris* on the hydroxyl radical scavenging effect than the same dose of mannitol, carbon tetrachloride-induced liver injury has a significant protective effect; oxygen free radical scavenging effect is not the same as the dose of ascorbic acid. Wang Qi (2002) reported that *Cordyceps militaris* can increase the activity of superoxide dismutase (SOD) and glutathione peroxidase (GSH2P) and reduce the content of lipid peroxides (LPO) in aged rats, organs and the body's aging.

12.2.3.5 Regulation of Immune System

Cordyceps, mainly in the immune organs, immune cells and immune molecules and other levels to play a role in enhancing the body's immune function. A lot of

research reports, typical of Zhu, S. and J. with different doses of FCM and DCM in 2013 after oral administration in 15 days, in the cyclophosphamide (CY) induced immunosuppressive mice; in vitro, spleen cell extraction and overlapping with CY from healthy mice, then cultured with different doses the FCM or DCM; Cordyceps polysaccharide (CMP) content of cordycepin, adenosine, total phenolic (TP) and total flavonoids (TF) on FCM and DCM were determined. FCM was significantly higher than DCM in the spleen and thymus index, spleen lymphocyte activity, macrophage function, promoting IL-2, IFN and in vivo and in vitro. The contents of CMP and TF in FCM were significantly higher than those in DCM. Conclusion: These results suggest that FCM is superior to DCM in enhancing immunity. Cui Xinying et al. (2004) reported that Cordyceps can significantly improve the weight of mice spleen and thymus, and the amount and phagocytic activity of peritoneal macrophages.

12.2.3.6 Endocrine and Anti Fatigue Effects

Cordyceps also has other effects, such as the role of androgen, can improve insulin secretion; Jia Jingming, etc. (2003) proved that the elimination of blood lactic acid and the occurrence of delayed fatigue also has a more significant role.

12.2.4 Artificial Cultivation

China is the first country of commercial cultivation of *Cordyceps militaris* production, Jilin province in 1986, so the tussah silkworm and silkworm as the host culture of *Cordyceps militaris* and natural Cordyceps, consistent fruiting body, opened a new era of China's artificial cultivation of *Cordyceps militaris*. Rice, millet or sorghum rice can provide some carbon source for the growth of Cordyceps.

Zhang Xianke (1997) successful use of millet and the appropriate nutrient solution to cultivate a normal sub entity. Li Yuan with rice as the basic medium added to the nutrient solution to cultivate normal stroma, only the formation of orange red mycelium agglomerate and millet instead of rice, nutrient solution on the normal formation of entity may play an important role. Liu Di (2004) proved that Cordyceps can make use of plant protein, but it is still an animal protein. In terms of culture conditions, the temperature to 15–25 °C is appropriate, cannot continue below 10 °C, also cannot be higher than 30 °C, otherwise it will lead to stagnant growth or dead mycelium. Humidity requirement between 90% and 60%. During the growth phase, the growth phase of the fruiting body needs to be as long as 10 12 h, and the growth period is about 45–60 days. The use of silkworm chrysalis and cicada pupa etc. insect host fruitbody production is another important way to cultivate. Artificial cultivation of *Cordyceps sinensis* has made great progress, but the problem is still a major factor restricting the development of the industrialization of Cordyceps, breeding fine strains will be one of the hot spots in the future.

12.3 *Cordyceps cicadae*

Commonly known as Cordyceps cicadae, belonging to the entomogenous fungi; cicada larvae are parasitic on Cordyceps fungus, cicada before eclosion, when the climatic conditions suitable to absorb the worm nutrition into mycelium, the ultimate parasite is mycelium completely accounted for and only a shell. The recovery time, and from the vegetative stage mycelium was gradually transformed into a sexual stage with reproductive function "cicadae spores", gradually from the top branches of "germination" shape of flowers, so called cicada.

12.3.1 Historical

As the Ming Dynasty Li Shizhen "Compendium of Materia Medica" (1590) and some other drugs Chinese ancient historical records: "cicada can cure epilepsy, yeti palpitations, work with chantui".

In addition to the classical medical works, there are many ancient medical records of Compound Cordyceps cicadae monographs, such as the Song Dynasty "Shengji Zonglu", "Xiao" etc.

12.3.2 Morphology and Structure

12.3.2.1 Morphological Structure

The morphological structure of the flower was mainly composed of (1) sclerotium; (2) the spore stalk beam (3) the cicada flower spore powder composed of three parts (Fig. 12.4).

12.3.2.2 Sclerotia

Sclerotia, the cicadas cicadas larvae infected with Cordyceps bacteria after the parasite, sclerotia formed by the three-tier structure, the outermost of the milky white called "bacteria was" structure, thickness 0.5 mm, top grade gold cicada Layer



Fig. 12.4 Medicinal Mushroom Cordyceps Cicadae. Being Composition by fruiting body like flower with orange color and Cicada

completely wrapped worm; the middle layer is the shell of the cicada larvae, the name of medicine in the "cicada"; the innermost layer of "mycelium" that is transformed from the cicada's nutrients. Therefore, nature has this three-tier structure and carry full of cicada flower spore powder Need for the cicadas is very rare. Sclerotia long kidney shape, slightly curved, about 2.5–3.5 cm, diameter 1–1.4 cm, the shape of cicada larvae. Rod body with 1–2 rod-shaped sub-blocks, also known as spore stems, long or curled, branched or unbranched, long 3–7 cm, diameter 3–4 mm, the original eco-egg white, dried milky white, Some are dark brown, the top slightly swollen, the surface of powdery cicada spore powder, the shape of flowers.

12.3.2.3 Spore Stem Beam

Also known as the fruiting body, sub-seat, born from the front of the host, fresh white, high 1.5–6 cm; handle branched or unbranched, diameter 0.1–0.2 cm.

12.3.2.4 Spore Powder

Cicada flower spore powder, which is the flower of the "seed" (bottom right), cicada flower spore powder has reproductive function, the conidial spore shape is rectangular egg-shaped, Slightly pointed at both ends, the size of (6–9) μm × (2–2.5) μm, often containing two oil balls, for the cicada flower spore oil, transparent colorless. Cicadaepora spore powder is from the sclerotium, spore stems stem from the active ingredient into, therefore, the flower of the cicada flower spores gathered the active ingredients of the essence of the flower, with anti-tumor effect.

Cicada other names have large caterpillar fungus, cicadas, cicada, worm flowers, etc., is the cicada nymph infection with cicada Paecilomyces Paecilomyces (Paecilomyces cicadae) after the formation of entomogenous fungi. This bacterium in 1838 by the Miquel named cicada cordis (Isaria cicadae). Since then there are a variety of homonyms. Such as Cordyceps cicadae, Isaria basili, Sphaeria sinclairii, and the like. Cicada Paecilomyces sexuality stage, is considered a large cicada (Cordyceps cicadae), large cicada common name Dujiao Long, sub-rod-shaped or horny, solitary or tufted, brown. Widely distributed in nature is cicada Paecilomyces (cicada), a large cicada rare.

12.3.3 Compositional Characteristics

Containing liver sugar, Cordyceps acid, a variety of alkaloids and ergosterol and so on. Japan isolated from the parasite part of the alkali-soluble polysaccharides of cicada, cicadae contains a large number of chitin and nitrogen, and its function to reduce rhabdomyolysis, and blocking the ganglion.

The taste: Gan; cold; non-toxic.

Herb source: for the ergot fungus Corynebacterium parvum spores stems stem beam, the great seat of the grass and a total parasitic worm body.

The chemical composition of the Chinese herbal medicine: the great cicadae fruit contains galactomannan (galactomannan), from D-mannose (D-mannose) and

D-galactose D-galactose) to 4:3 ratio composition. Part of the parasite polysaccharide CI-5N, CI-P and CI-A.

Cicadas is a ergot fungus cicada cicada Parasitic nymphae nymphs after the complex is *Cordyceps sinensis* similar to the Cordyceps; and originating in the Hengduan Mountains, Tianmu Mountain infected insects for the wild cicada cicadas called cicadas Jin Chan or large cicada grass (see Baidu library Lvdi "Jin Chan Flower"). The earliest in the Northern and Southern Dynasties Lei Lei's "Lei Gong gun on" there is processing cicada flower records. Song of the Tang cautious "Zheng Materia Medica", and the Ming Dynasty Li Shizhen "Compendium of Materia Medica" and after the pharmacopoeia has documented efficacy. Cicada spent more than 800 years history of *Cordyceps sinensis*, but the natural cicada is very scarce, wild Chanchan more rare and precious, which limits the extensive use of cicada.

12.3.4 Pharmacological Effects

1. Anti-tumor effect: Anti-tumor effect, cicada anti-tumor, prevent cancer cell proliferation and metastasis is achieved by improving the patient's immune system, "China Cancer Treatment and Protection Network," Lvdi director of cancer at home and abroad and authoritative experts believe that the root cause of cancer is the decline in immune function. The immune system is the body's own natural barrier, can produce anti-cancer and other physiological balance to maintain the body and stable immune protection. It can improve the tolerance of radiotherapy and chemotherapy to the patients receiving radiotherapy and chemotherapy. It can eliminate the severe pain of cancer, reduce or eliminate the side effects of leukopenia, alopecia, loss of appetite and vomiting caused by radiotherapy and chemotherapy. Quality of life, to extend life. Large cicada polysaccharide (galactomannan) also has anti-tumor effect.
2. The role of the central nervous system: The role of the central nervous system mice intraperitoneal injection of natural cicada or artificial culture dilute alcohol extract can significantly reduce their autonomy, prolonged pentobarbital sodium and chloral hydrate caused by sleep time, improve Sublingual hypnotic volume of pentobarbital sodium in mice; prolong the latency of convulsions in mice that are stimulated by central stimulant thiazole and pentylenetetrazole. The chemical stimulation and hot plate method proved: the analgesic effect of the two obvious. To the normal and yeast-induced intraperitoneal injection of heat, with a significant cooling effect. Additional data further prove that cicadae and its artificial culture, there is significant analgesic, sedative and antipyretic effect.
3. Toxicity: Acute toxicity experiments showed that: natural cicadae alcohol extract mice gavage 60 g/kg, observed 72 h, 20 mice without death, only after administration of animals decreased activity, 24 h returned to normal. Intraperitoneal

injection of LD50 was 12.5 ± 2.1 g/kg, the toxicity of the performance of writhing, reduced activity, difficulty breathing until death. The results of subacute toxicity test showed that the rats were given intragastric administration of 1 g/kg, 3 g/kg and 9 g/kg respectively for 28 days. The results showed no abnormal changes in blood routine, liver and renal function and electrocardiogram. Heart, liver, spleen, lung, kidney and other important organ pathology examination also no obvious abnormal changes.

12.3.5 Characterstics

1. Corynebacterium parachyposis: This product from the parasite and its head out of the composition of the spore stalk. Body length oval, slightly curved, about 3 cm, diameter 1–4 cm, the surface of brown, mostly gray mycelium coating, the head of the cluster bundle spores. Sporophyll-branched or unbranched, 1.6–6 cm long, subequal to solid and sessile; robust minister elliptic, elliptic or spindle-shaped, 5–8 mm long, 2–3 mm in diam. Diameter 1–2 mm, brown to dark brown. Crisp, easy to break, the body is full of white or white soft material like. Gas slightly fragrant, tasteless.
2. Large cicada: This product from the worm and its front-end sub-base composition. 1–2 cm in length, branched or unbranched, 3–7 cm long, brown; head swollen, tapering, 4–6 mm long, 6.5–7 mm in diameter, with small dots on the surface Of the orifice), the stem diameter of 4–5 mm. Worm body white, the body covered with white hyphae. Crisp, easy to break. Gas micro, tasteless.
3. Corynebacterium coryneform bacterium sporophyte bottle-shaped, central enlargement, terminal tapering or suddenly narrow, long 5–8 μm, diameter 2–3 μm often clustered in the bundle of silk, shaped like a petal. Conidia oblong, spindle-shaped or narrow kidney-shaped, long 5–14 μm, diameter 1.8–3.5 μm, with 1–3 fat droplets.
4. Large cicada grass seat head cross-section: subcapsular shell embedded in the sub-seat, bottle-shaped, long 350–540 μm, diameter 125–300 μm; subcapsular cylindrical, flat spherical cap length 262.5–378 μm, diameter 6.2–9.1 μm; ascospores slender filamentous, multiple septum, ruptured rectangular small segment length 3.5–5.2 μm, diameter 1.7–2.6 μm.

12.3.6 Functionality

Studies at home and abroad have shown that cicada has many functions, such as improving immunity, resisting fatigue, protecting kidney, improving sleep, resisting tumor, protecting liver, resisting radiation and eyesight, and is a magic ancient Chinese medicine. The contents of arsenic, mercury, lead and other toxic heavy metals were similar to those of natural *Cordyceps sinensis*, but the contents of arsenic, mercury, lead and other heavy metals were not detected in artificially cultured

C. sinensis with 2.18% mannitol, 21.73% polysaccharide, 19.76% amino acids, 0.005% cordycepin and 0.05% Out, which is safer than natural *Cordyceps sinensis*. Therefore, cicadas can be used as a substitute for *Cordyceps sinensis*, the same can achieve the role of nourishing health.

12.3.6.1 Tonic

Chen Wanqun et al. reported that the results show that cicada and a variety of Cordyceps in the main components of amino acids similar to the content of more consistent. Scholars have recognized a variety of amino acids is one of the material basis for tonic strong, pharmacological experiments show that a variety of Cordyceps and Cordyceps amino acids have different levels of beneficial effects.

12.3.6.2 Anti-Fatigue

Cicada spent decoction can significantly extend the swimming time of mice, significantly increased atmospheric hypoxia and high temperature conditions in the survival time, prove that cicada has anti-fatigue and anti-stress.

12.3.6.3 Hypnosis

Cicada spent mice 1 h after the determination of the number of autonomous activities within 10 min significantly less than the control group; cicada can significantly prolong the sleep time of mice, shorten the disappearance of pentobarbital sodium righting reflex time; cicadas can also increase Mice in the unit time to sleep. This shows that cicada has better sedative and hypnotic effects. At the same time studies have shown that artificial culture and the role of natural cicada close.

12.3.6.4 Analgesia

The results showed that cicadae had significant effects on the chemical and thermal pain of mice, and the effects of cicadella on chemical and thermal pain in mice were significant Inhibition. It is proved that the cicada has good antipyretic and analgesic effects. This experiment and Chen Zhu and others experiments have proved that cicadae artificial culture products also have the same effect.

12.3.6.5 Immunization

The cicadae strains were artificially fermented to produce cicadae hyphae, and the polysaccharides from the cicadae were extracted. The *Cordyceps sinensis* polysaccharide was used as the positive control and the polysaccharides of Grifola frondosa as the reference. The lymphatic transformation test, Ea and E roses test, Specific immune rosette test (macrophage phagocytosis test, anti-sheep red blood cell (SRBC) antibody titer test, the results show that the polysaccharide has a significant role in improving the immune function.

Shanghai University of Traditional Chinese Medicine Longhua Hospital Professor Chen Yiping, director of clinical Rudy and other clinical application confirmed that cicadas has reduced blood, urine creatinine, increased endogenous creatinine clearance rate, improve serum protein content and reduce urinary protein excretion and other functions. Therefore, early and mid-term efficacy of patients

with chronic renal insufficiency. After further study confirmed: cicadae renal tubulointerstitial lesions have a good effect, can protect the renal tubular cells Na + -K + -ATPase, reduce cell lysosomes and lipid peroxidation damage, improve renal hemodynamics, Reduce endothelial cell damage and blood clotting. So that can improve kidney function cicada.

12.3.6.6 Cicada Appropriate Crowd

Cicada appropriate crowd: dyspnea, pulmonary fibrosis, hardening of the liver, all kinds of liver disease, kidney disease, heart failure, impotence, cold, dry skin, dirty impatient, insomnia, cancer, metabolic syndrome, lupus erythematosus. Inflammation, prostatitis, colds and other low immunity, the elderly frail, postpartum physically weak and sub-health state is a rare and useful nutritional supplements.

12.3.6.7 Allergy crowd

Cicadas is not suitable for the crowd: patients with rheumatoid arthritis should be reduced taking children, pregnant women and lactating women, fever, cerebral hemorrhage crowd should not eat, with real fire or evil winners should not be used.

Jinchan flower sweet, cold, with Bugan eyesight role, for floaters, cataracts, night blindness, blurred vision and so on.

12.3.7 Species Distribution

Cicadas are mainly found in China's Sichuan, Jiangsu, Zhejiang, Fujian, but Anhui, eastern Yunnan Province.

The land is distributed. In Zhejiang, the growth of bamboo hills, 80–500 m above sea level, gentle terrain, canopy density is higher, loose soil, humidity, ground covered with litter, and often bamboo forest activities of certain woodland, Generally can be taken to the cicada. On the contrary, the steep slope, Dicranopteris dichotoma, thatched, soil compaction, there is little occurrence of cicada. On the source of the three rivers in Yunnan Province, the results of the study, more than 2500 m above sea level, cicadas disappeared. The broad-leaved forest below 2500 m in altitude or the Cyclobalanopsis glauca, Castanea henryi, Pinus yunnanensis, Abies and other needle and broadleaf mixed forest, canopy density, soil loose, litter layer thickness of some forest land can be collected Cicada specimens. The host is mostly small cicadas and mantis. Pure coniferous forest found no cicada.

12.3.8 Growth Habit

Cicada flammata, Platyleura kaempferi, Cicadatra shaluensis, Mogannia conica, Cymbidium cicadae, Cicadellidium cicadae, Cicadellidium cicadae, Cicada flammata, Oncotympana ella and Hyalessa ronsnana, distributed in the southern



Fig. 12.5 The environment of Medicinal Mushroom Cordyceps Cicadae growth. The Medicinal Mushroom Cordyceps Cicadae is the worm of Cicada being infected by *Cordyceps militaris* and the fruiting body being grow out of the ground

provinces of China. Bamboo cicada common in the bamboo-producing areas, is an important host of cicada Paecilomyces. This insect 6 years one generation, each generation of 5 years old, perennial living in the soil to the oldest nymphs most susceptible to disease. When the temperature 18–24 °C, relative humidity >80% of the warm and humid season, in the shallow soil activity of the nymphs, contact with infected soil infection. To the next year 6, 7 months, the appropriate temperature and humidity, the incidence of death in front of the worm body to grow pale yellow or egg yolk spores stem, breaking the topsoil, reaching the ground, forming our common cicada (Fig. 12.5).

12.4 Liangshan Cordyceps

Liangshan Cordyceps look like the child is more than branches or single branches, slender and hard, upright and tortuous, born out by the host mouth.

12.4.1 Morphological Characteristics

High 20–30 cm, coarse 1.5–2.3 cm. Head cylindrical or rod-shaped, brown, dark brown, the top of the infertile tip of the extension, thick 2–2.3 mm, with false parenchyma cortex (Fig. 12.6). Peritheal shell oval or oval, 400–740 μm × 300–450 μm, dark brown, the surface of Health, protruding days were Southern Star fruit sequence. The sub-shells are cylindrical, 260–480 μm × 8–12 μm. Ascospore transparent or yellowish, linear, worm-like, multi-septal, 160–350 μm × 2.5–3.5 μm, was broken, broken after the spores of small segments, each about 10–20 μm × 2.5–3.5 μm. Sub-shell under the shell handle and more branches, fresh brown or straw color, even a single elongated erect branches. Host mycelium, fresh milky white.



Fig. 12.6 Morphology of Cordyceps liangshanensis Zang, Liu et Hu. Being Composition by fruiting body with orange color and worm



Fig. 12.7 The environment of Cordyceps liangshanensis Zang, Liu et Hu. Growth. The Cordyceps liangshanensis Zang, Liu et Hu is the worm being infected by *Cordyceps militaris* and the fruiting body being grow out of the ground

12.4.2 Ecological Habits

Parasitic on Lepidoptera larvae, more common in the area below 1500 m above sea level, especially in the bamboo plexus in the worm body (Fig. 12.7).

12.4.3 Distribution Area

Sichuan, Guizhou, Yunnan, etc., for our endemic species.

12.4.4 Economic Uses

Edible fungus. In the goods. Sichuan area market farmers to sell this on behalf of the authentic Cordyceps. Because of fiber quality, so the quality inferior times. The highest

value of the domestic medicinal Cordyceps produced in Tibet, Nagqu, Qamdo and Qinghai Yushu, Guoluo these origin, which originated at an altitude of 3000–4000 m line, anti-cancer natural cordycepin and the highest content of natural amino acids.

12.4.5 Artificial Cultivation

Sichuan Liangshan Yi Autonomous Prefecture drug testing had to separate the hypha in the potato agar culture medium, hyphae community white, stunted, not by the seat.

12.4.6 Medicinal Value

For virtual breath labor cough.

12.5 Gurney Cordyceps

Gurney Cordyceps, also known as incense stick Cordyceps, Hawkes Cordyceps, parasitic in the earth bat moth insect larvae. Ovary born from the head of the host, handle white, the top general gray to grayish black, long oval to columnar, solitary, bifurcation or clusters of students, usually 8–22 mm × 5–8 mm, mature and Clear boundaries of the handle, no infertility top. Ascus capsule oval-shaped or ampoule-shaped, buried, mature exposed orifice. Distributed in Guizhou, Hunan, Guangdong, Yunnan and other places.

12.5.1 Introduction

Gurney Cordyceps is a summer born in the broadleaf forest buried in the larvae of Lepidoptera insects in 1983, Gurney Cordyceps first reported in China, Liang Zongqi and other strains of its separation, an asexual to determine the nutritional composition of pharmacological Experiments and liquid fermentation and other aspects made a lot of research work, indicating that Gurney Cordyceps in improving the body immunity, promote sleep and enhance memory, analgesia and so has a very important role. With the continuous improvement of experimental technology, Gurney Cordyceps still has great potential for applied research. In this paper, the biological characteristics, chemical constituents and pharmacological effects of *Cordyceps sinensis* were summarized, with a view to further understanding of the biological characteristics of *Cordyceps sinensis* and related products to provide the basis for in-depth research and development.

12.5.2 Morphological Characteristics

Gurney Cordyceps general length 10 ~ 90 mm, coarse 5 ~ 6 mm. Gurney worm grass seat from the head of the host to give birth, solitary, bifurcation or clusters of



Fig. 12.8 Cordyceps gunnii (Berk.) Berk. Being Composition by fruiting body with orange color and worm

students. Generally longer than the worm body 4 ~ 14 cm, base white, coarse, up gradually fine. The head is generally gray to gray-black, long oval to cylindrical, forming a sub-head (Fig. 12.8). Immature Gurney Cordyceps pale yellow surface, sub-seat inside the full, sub-seat head white. Mature Gurney caterpillar fungus surface brown, sub-seat filling gradually into a hollow, the entire sub-head rough, brown, length 1.5 ~ 2.5 cm. There are many sub-capsule shell formation of tiny particles, ripe Gurney grass seat and handle the clear boundaries, ascus shell oval-shaped or ampoule-shaped, 700 ~ 9l0 μm × 200 ~ 300 μm, buried, mature exposed orifice.

12.5.3 Chemical Composition

Cordyceps sinensis contains seven kinds of standard nucleosides in the three, namely adenosine, cytidine, uridine. The contents of adenosine and uridine in the mycelia of *Cordyceps sinensis* were two to three times higher than those of *Cordyceps sinensis*, and the content of adenosine and uridine in its sub-seat was higher Dead body. Cordycepin (3-deoxyadenosine) is one of the important physiologically active substances of Cordyceps. Cordycepin can inhibit the growth of Bacillus subtilis, it is very low in the initial stage of mycelial growth, with the mycelial growth of cordycepin content was significantly improved.

12.5.4 Pharmacological Effects

12.5.4.1 Analgesic Effect

Zhu Zhenyuan and other active substances in the Gurney Cordyceps test, proved analgesic substances in the free amino acid content is low, and high levels of hydrolyzed amino acids, indicating that the analgesic active ingredient is not free amino acids, but polypeptide material, and in the polypeptide chain is acidic. In the study of drug dependence of Gurney's analgesic active ingredient, the mouse tail-tail test and the rat attack test showed that the antitussive component of Gyne Cordyceps had no three kinds of morphine-dependent drugs, significantly reduced the

spontaneous activity time of mice, Can prolong the sleep time of pentobarbital sodium model mice, and show obvious analgesic effect.

12.5.4.2 Improve Human Immunity

Cordyceps can ensure and enhance macrophage phagocytosis, increase the percentage of phagocytosis, the phlegm phagocytosis coefficient was significantly increased, and to ensure that T lymphocytes from injury, enhance cellular immune function and enhance liver function. Promote metabolism. Cordyceps can also improve immune and hematopoietic function, so that the peripheral blood and spleen lymphocytes enhanced, and spleen cells to produce interleukin-enhanced ability to increase the natural killer cell activity and promote hematopoietic cell proliferation. The immune or hematopoietic function of cancer and other diseases have significant role in adjuvant therapy.

12.5.4.3 Anti-Ultraviolet Radiation

Meng Xiangjian and other research on the ancient Chinese caterpillar fungus showed that Gurney Cordyceps produced a substance that can protect against ultraviolet radiation. The components in the 80 °C constant temperature bath application does not deactivate, and the application of anti-ultraviolet radiation, the substance content in the mycelium and fruiting bodies in the higher, less in the culture medium, for what components to be further studied.

12.5.4.4 Anti-Tumor Effect

Cordyceps can inhibit cancer cell fission, prevent cancer cell proliferation, significantly increased in vivo T cells, macrophages phagocytosis, so that they greatly enhance the ability of cancer cells to destroy. Experiments show that edible Cordyceps 1 month after the tumor inhibition rate of 629/6 or more. Studies have shown that Cordyceps in vitro culture of KB cells and Hela cells have a special inhibition. Xu Renhe and other studies have shown that administration of *Cordyceps sinensis* alcohol extract can significantly enhance NK activity in mice and inhibit the formation of lung cancer clones, and can partially antagonize the inhibitory effect of Cy on NK activity in mice.

12.5.4.5 Anti-Aging Effect

In the medical profession with anti-aging seven categories of substances, Cordyceps covers five categories, namely polysaccharides, amino acids, peptides (proteins), nucleic acids and vitamins (the other two are flavonoids and gland glycosides). Li Shufang and other in the use of Paecilomyces Gurney on aging rats blood and tissue lipid peroxides and superoxide dismutase activity of the study showed that: P. gurnii can reduce the aging rats plasma, liver and Brain tissue LPO content; enhance red blood cells, liver and brain SOD activity, thus play the role of anti-aging.

12.5.4.6 To Promote the Role of Sleep and Memory

Gurney Cordyceps contains polysaccharides, amino acids, vitamins and other nutrients, the brain can provide sufficient energy and nutrition to ensure the normal

functioning of the brain; also contains excitatory amino acids such as glutamic acid, aspartic acid, glycine, etc. are Selectivity and transmitter receptors, can activate the NMI) _R channel to open channel Ca Ca, Ca intracellular increase in regulation of excitatory synaptic transmission and development may be involved in learning and memory synapses.

12.5.4.7 Protection of Acute Ischemic Brain Injury

Xiong Zhengmei and other studies have shown that Goni Cordyceps anomorphic—Paillus penicillinum on acute ischemic brain damage in rats have a protective effect.

12.5.5 Identification of Medicinal Materials

Character identification. This product is the parasite and its head out of the sub-blocks. Worms like silkworm, 3–4 cm long, 4–5 mm in diameter, head reddish yellow or purple-black, white surface, there are 20–30 links, near the head has three pairs of feet, tail one, four pairs, the valve punctate, black, scrape off the outer white membrane, showing brown or chestnut brown body worm skin; crisp, easy to break, section flat, yellow-white. Apex rounded, 1–1.2 cm in length, 3–6 mm in diam., Tan brown, with more than 2 mm in diameter, Gray or gray-black, with vertical lines; crisp, easy to break, loose section or empty deflated. Gas smell, taste slightly salty (sclerotia) or light (sub-seat). Sub-seat cross-section: subcapsular shell embedded in the sub-seat, flask-shaped or sole shape, length 325–585 μm, diameter 65–156 μm; subcapsule length 304–398 μm, 3–5 μm in diameter, ascospores linear, 182–325 μm, diameter 1.5–2 μm, transverse septum was not obvious; wall mycelium arranged closely; mycelial mycelium arranged loose.

12.5.6 Culture Method

In the culture of Golgi Cordyceps culture medium to consider: nitrogen source, carbon source, water, constant and trace elements and other nutritional requirements. ① carbon source: the three sugar, polysaccharides and other carbon sources, the potato powder as carbon source, the highest biomass, and the ancient Cordyceps mycelium is pure white; ② nitrogen: inorganic nitrogen (ammonium chloride, nitric acid Sodium and ammonium sulfate, etc.) and organic nitrogen (peptone, yeast powder, beef extract, etc.), the experimental analysis of the best nitrogen source is NaN03; ③ trace elements: the experimental concentration of trace elements in the trial of the ancient The growth of M. cordyceps mycelia was not significantly affected.

The liquid fermentation was carried out by inoculating the spores of Paecilomyces lucidum spores in I and II medium, respectively. C after 15 days of standing and drying. After fermentation in the first-stage seed tank for 2 days, the fermentation was carried out by using the deep fermentation method. Into the secondary tank fermentation 2 days harvest, filtration, low temperature drying mycelium.

12.5.6.1 Solid Culture Method

In the cultivation of Gurney Cordyceps, pay attention to their life history and the conditions required for growth, such as temperature, humidity and other factors. In the cultivation of Paecilomyces lucidum, when the culture medium was inoculated with the spore suspension of Paecilomyces lucidum, the culture was carried out after culturing at different temperature for 15 days, and the culture was taken out after 40 days low temperature drying. Fu Lan, etc. in the deep fermentation of *Cordyceps sinensis* mycelium medium conditions, to determine the potato 20%, peptone 1%, glucose 2%, 0.1% magnesium sulfate, potassium dihydrogen phosphate 0.05% and 0.1% The optimum fermentation conditions were as follows: temperature 25 ~ 26 °C, rotation speed 150 r/min, pH 6.5, shaking culture 120 h, 20 h fermentation tank 66 h F73.

12.5.7 Development Prospects

At this stage of the study of *Cordyceps sinensis* has made gratifying achievements. Gurney Cordyceps with enhance immunity, promote sleep and enhance memory, analgesic and other effects, so in medicine and health care products development has a high value. Among them, Guizhou on the development of ancient Chinese caterpillar fungus has been walking in the forefront of China, Guizhou Chitianhua Group Co., Ltd. and Guizhou University to develop ancient Nigeria Cordyceps resources, and now has achieved great success, has developed the A Fu Le Cordyceps wine and tea And other ancient Nigeria Cordyceps products. Guizhou Renhuai Maotai Hanfang Liquor Industry Co., Ltd., has developed a variety of alcoholic liquor such as Emperor Maojiu and Fu Guo Liquor. It uses aging liquor and joins valuable raw materials such as *Cordyceps sinensis*. The main use of ancient Nigeria Cordyceps is rich in nutrients, Gurney Cordyceps and wine with wine brewing, and its products have a certain therapeutic value. However, until now, the study of bioactive compounds of *Cordyceps militaris* has not been deep enough. In this case, the development of new *Cordyceps sinensis* and other Chinese herbal medicine has important significance, such as compound Chinese caterpillar fungus capsule, Compound Chinese caterpillar fungus and so on, take the traditional Chinese medicine as the medicine lead, directs the Cordyceps active substance to give full play to its medicinal effect, is develops the ancient Chinese Cordyceps a new way. However, because Goni Cordyceps has a lot of biological activity, it should increase its independent research and development, so as to give full play to its unique effect.

In short, the study of *Cordyceps sinensis* need to increase its efforts to clear its pharmacological properties and the correlation between the active substances to accelerate the transformation of research results, and shorten its cycle. Now the understanding of *Cordyceps sinensis* and Cordyceps research is not so in-depth and thorough, but the study found that some active ingredients of Cordyceps *Cordyceps sinensis* is much higher than the *Cordyceps sinensis*, how to develop and use this valuable resource to play its pharmacological effects, which Gurney Cordyceps for

researchers is undoubtedly put forward higher requirements. Cordyceps on the depth of research and its series of products to develop, to play a more important role in human beings.

12.5.8 Planting Techniques

Bacteria: strains are mostly from the nature of the ancient Chinese caterpillar fungus, according to conventional isolation and culture. According to the research, Cordyceps fungus sexual occurrence process, the need for a certain degree of humidity to meet the complex physiological changes in the required water, and the temperature and humidity there is a correlation between, in a certain humidity, the temperature changes slowly or more constant, Is not conducive to extract the fruiting body. Sexual occurrence must be through the low temperature and variable temperature treatment Pathways: Gurney Cordyceps is Cordyceps spores contact larvae infection death, in order to grow fruiting bodies. It was observed that the host 4–5 instar larvae of the highest infection rate. Mature larvae rarely infected, three instar larvae are not infected. How to grasp the timing, the average person is also very difficult to grasp. In the dip problem, the artificial breeding of insects better conditions, the worm is too strong, strong antibacterial, difficult to infection. Conditions are poor, then the invasion of insects cause death, both of which will fail.

Mimic the origin of the ecological environment: that is, at an altitude of 3500–5000 m on the mountains with the temperature, humidity, light, soil, vegetation and other conditions, which is generally difficult to do. It is also the above reasons, most people cannot plant Gurney absolute Cordyceps.

12.6 Large Capsule Cordyceps

Large capsule Cordyceps, traditional Chinese medicine name. For the ergot fungi large group of Cordyceps ophioglossoides (Ehrenb.) Link of the seat. Distributed in Jiangsu, Guangxi, Sichuan, Yunnan and other places. With blood, bleeding, menstruation effect. Commonly used in the blood bank, irregular menstruation.

12.6.1 Morphological Characteristics

Large capsule Cordyceps: also known as Cordyceps trees, large group capsule grass. Sub-base by the root, multi-branched hyphae fixed in the soil on the host, the ground part of the high 2–8 cm. Shank coarse 1–2.5 mm, less branched, dark green to purple-brown, with vertical lines. Head oval, obovate to rod-shaped, 5–13 mm long, 3–5 mm thick, dark brown, dry nearly black (Fig. 12.9). Ascus shell oval, (600–650) μm × 300 μm, orifice protruding (300–400) μm × (7–8) μm. Spore linear, transparent colorless, with most of the diaphragm, mature to break into (3–4) μm × (2–2.5) μm small segment.



Fig. 12.9 Cordyceps ophioglossoides. Being Composition by fruiting body with orange color and worm

12.6.2 Growth Environment

Parasitic in bamboo or oak forest loose soil under the large group of bacteria (Elaphomyces granulatus Fr.) on the fruiting bodies. Distributed in Jiangsu, Guangxi, Sichuan, Yunnan and other places.

12.6.3 Herbal Character

Seat length of 2–6 cm, the roots of mycorrhizal residual root. Head oval, obovate or rod-shaped, 5–13 mm long, 3–5 mm in diameter, dark brown or dark brown; handle diameter 1–2.5 mm, less branched, dark green, with vertical lines. Crisp, easy to break, section dark brown. Gas slightly fishy, tasteless.

Further reading

Ahn YJ, Park SJ, Lee SG et al (2000) Cordycepin: selective growth inhibitor derived from liquid culture of *Cordyceps militaris* against Clostridium spp. J Agric Food Chem 48(7):2744–2748

Cannon PF, Kirk PM (2007) Fungal families of the world. CabinetMaker:456

Chang Y, Hsu WH, Lu WJ et al (2015) Inhibitory mechanisms of CME-1, a novel polysaccharide from the mycelia of *Cordyceps sinensis*, in platelet activation. Curr Pharm Biotechnol 16(5):451–461

Chen Y, Wang W, Yang Y et al (1997) Genetic divergence of *Cordyceps sinensis* as estimated by random amplified polymorphic DNA analysis. Acta Genet Sin 24(5):410–416

Chen Y-C, Huang Y-L, Huang B-M (2005) *Cordyceps sinensis* mycelium activates PKA and PKC signal pathways to stimulate steroidogenesis in MA-10 mouse Leydig tumor cells. Int J Biochem Cell Biol 37(1):214–223

Cho HJ, Cho JY, Rhee MH et al (2007) Inhibitory effects of cordycepin (3´-deoxyadenosine), a component of *Cordyceps militaris*, on human platelet aggregation induced by thapsigargin. J Microbiol Biotechnol 17(7):1134–1138

Choi HN, Jang YH, Kim MJ et al (2014) *Cordyceps militaris* alleviates non-alcoholic fatty liver disease in mice. Nutr Res Pract 8(2):172–176

Das SK, Masuda M, Hatashita M et al (2008) A new approach for improving cordycepin productivity in surface liquid culture of *Cordyceps militaris* using high-energy ion beam irradiation. Lett Appl Microbiol 47(6):534–538

Hong T, Cui LK, Wen J et al (2015) *Cordycepin* protects podocytes from injury mediated by complements complex C5b-9. Sichuan Da Xue Xue Bao Yi Xue Ban 46(2):173–178. 227

Hur H (2008) Chemical Ingredients of *Cordyceps militaris*. Mycobiology 36(4):233–235

Jagger DV, Kredich NM, Guarino AJ (1961) Inhibition of Ehrlich mouse ascites tumor growth by cordycepin. Cancer Res 21(2):216–220

Jeong MH, Lee CM, Lee SW et al (2013) Cordycepin-enriched Cordyceps militaris induces immunomodulation and tumor growth delay in mouse-derived breast cancer. Oncol Rep 30(4):1996–2002

Jing Y, Cui X, Chen Z et al (2014) Elucidation and biological activities of a new polysaccharide from cultured *Cordyceps militaris*. Carbohydr Polym 102:288–296

Jing Y, Zhu J, Liu T et al (2015) Structural characterization and biological activities of a novel polysaccharide from cultured *Cordyceps militaris* and its sulfated derivative. J Agric Food Chem 63(13):3464–3471

Jong-Ho KOH, Kwang-Won YU, Hyung-Joo SUH, Yang-Moon CHOI, Tae-Seok AHN (2002) Activationofmacrophages and the intestinal immune system by an orally administered decoction fromcultured mycelia of *Cordyceps sinensis*. Biosci Biotechnol Biochem 66:407–411

Koh J-H, Kim K-M, Kim J-M et al (2003) Antifatigue and antistress effect of the hot-water fraction from mycelia of *Cordyceps sinensis*. Biol Pharm Bull 26(5):691–694

Kuo HC, Su YL, Yang HL et al (2005) Identification of Chinese medicinal fungus Cordyceps sinensis by PCR-single-stranded conformation polymorphism and phylogenetic relationship. J Agric Food Chem 53(10):3963–3968

Lee DH, Kim HH, Lim DH et al (2015a) Effect of *Cordycepin*-Enriched WIB801C from *Cordyceps militaris* Suppressing Fibrinogen Binding to Glycoprotein IIb/IIIa. Biomol Ther 23(1):60–70

Lee H, Kim YJ, Kim HW et al (2006) Induction of apoptosis by *Cordyceps militaris* through activation of caspase-3 in leukemia HL-60 cells. Biol Pharm Bull 29(4):670–674

Lee JS, Kwon DS, Lee KR et al (2015b) Mechanism of macrophage activation induced by polysaccharide from *Cordyceps militaris* culture broth. Carbohydr Polym 120:29–37

Liang HH, Cheng Z, Yang XL et al (2008) Genetic diversity and structure of *Cordyceps sinensis* populations from extensive geographical regions in China as revealed by inter-simple sequence repeat markers. J Microbiol 46(5):549–556

Liao Y, Ling J, Zhang G et al (2015) *Cordycepin* induces cell cycle arrest and apoptosis by inducing DNA damage and up-regulation of p53 in Leukemia cells. Cell Cycle 14(5):761–771

Lu Q, Mei W, Luo S et al (2015) Apoptosis of Bel-7402 human hepatoma cells induced by a ruthenium(II) complex coordinated by *cordycepin* through the p53 pathway. Mol Med Rep 11(6):4424–4430

Mao XB, Zhong JJ (2004) Hyperproduction of cordycepin by two-stage dissolved oxygen control in submerged cultivation of medicinal mushroom *Cordyceps militaris* in bioreactors. Biotechnol Prog 20(5):1408–1413

Pan BS, Wang YK, Lai MS et al (2015) *Cordycepin* induced MA-10 mouse Leydig tumor cell apoptosis by regulating p38 MAPKs and PI3K/AKT signaling pathways. Sci Rep 5:13372

Stensrud Q, Schumacher T, Shalchian-Tabrizi K et al (2007) Accelerated nrDNA evolution and profound AT bias in the medicinal fungus *Cordyceps sinensis*. Mycol Res 111(4):409–415

Wang L, Xu N, Zhang J et al (2015a) Antihyperlipidemic and hepatoprotective activities of residue polysaccharide from *Cordyceps militaris* SU-12. Carbohydr Polym 131:355–362

Wang Y, Liu D, Wang W et al (2015b) *Cordyceps sinensis* polysaccharide inhibits PDGF-BB-induced inflammation and ROS production in human mesangial cells. Carbohydr Polym 125:135–145

Wei HP, Ye XL, Zhang HY et al (2008) Investigations on cordycepin production by solid culture of *Cordyceps militaris*. Zhongguo Zhong Yao Za Zhi 33(19):2159–2162

Wen L, Tang YL, Yin QF et al (2005) Assays on nutrient and effective ingredients in different parts of *Cordyceps militaris*. Zhongguo Zhong Yao Za Zhi 30(9):659–661

Xiang F, Lin L, Hu M et al (2016) Therapeutic efficacy of a polysaccharide isolated from *Cordyceps sinensis* on hypertensive rats. Int J Biol Macromol 82:308–314

Xie CY, Gu ZX, Fan GJ et al (2009) Production of cordycepin and mycelia by submerged fermentation of *Cordyceps militaris* in mixture natural culture. Appl Biochem Biotechnol 158(2):483–492

Xiong C, Xia Y, Zheng P et al (2013) Increasing oxidative stress tolerance and subculturing stability of *Cordyceps militaris* by overexpression of a glutathione peroxidase gene. Appl Microbiol Biotechnol 97(5):2009–2015

Yang J, Zhang W, Shi P et al (2005) Effects of exopolysaccharide fraction (EPSF) from a cultivated *Cordyceps sinensis* fungus on c-Myc, c-Fos, and VEGF expression in B16 melanoma-bearing mice. Pathol Res Pract 201(11):745–750

Yang JL, Xiao W, He HX et al (2008) Molecular phylogenetic analysis of Paecilomyces hepiali and *Cordyceps sinensis*. Acta Pharm Sin 43(4):421–426

Yang X, Li Y, He Y et al (2015) *Cordycepin* alleviates airway hyperreactivity in a murine model of asthma by attenuating the inflammatory process. Int Immunopharmacol 26(2):401–408

Zhang Y, Xu L, Zhang S et al (2009) Genetic diversity of *Ophiocordyceps sinensis*, a medicinal fungus endemic to the Tibetan Plateau: implications for its evolution and conservation. BMC Evol Biol 9(1):290

Zheng P, Xia Y, Xiao G et al (2011a) Genome sequence of the insect pathogenic fungus *Cordyceps militaris*, a valued traditional Chinese medicine. Genome Biol 12(11):R116

Zheng Z, Huang C, Cao L et al (2011b) Agrobacterium tumefaciens-mediated transformation as a tool for insertional mutagenesis in medicinal fungus *Cordyceps militaris*. Fungal Biol 115(3):265–274

Zheng Z, Jiang K, Huang C et al (2012) *Cordyceps militaris* (Hypocreales: Cordycipitaceae): transcriptional analysis and molecular characterization of cox1 and group I intron with putative LAGLIDADG endonuclease. World J Microbiol Biotechnol 28(1):371–380

Zhou XW, Wang XF, Li QZ (2012) Expression and characteristic of the Cu/Zn superoxide dismutase gene from the insect parasitizing fungus *Cordyceps militaris*. Mol Biol Rep 39(12):10303–10311

Zhu SJ, Pan J, Zhao B et al (2013) Comparisons on enhancing the immunity of fresh and dry Cordyceps militaris in vivo and in vitro. J Ethnopharmacol 149(3):713–719

Zhu ZY, Liu XC, Dong FY et al (2016) Influence of fermentation conditions on polysaccharide production and the activities of enzymes involved in the polysaccharide synthesis of *Cordyceps militaris*. Appl Microbiol Biotechnol 100(9):3909–3921

Application of Recombinant Insect Products in Modern Research: An Overview

13

Mohd Yusuf

Abstract

Biotechnology enables the genetic engineering through gene modification, broadening the range of natural products, as far production and application of transgenic products, such as implant coatings, scaffolds for tissue engineering, wound dressing devices, as well as drug delivery systems. In the present scenario, recombinant technology including the expression of DNA and gene modification or simple genetic manipulation to several host organisms, involving bacteria, yeast, plants, insect cells, mammalian cells, and transgenic animals seems to have tremendous and promising future research opportunities. In this chapter, an attempt is made onto modern research initiatives using recombinantly produced insect products and applications.

13.1 Introduction

Recombinant DNA technology is one of the most emerging and innovative technologies applied commonly for medicine, agriculture, and industry to produce new genetically engineered products that are of high specific value. Interestingly, several expression systems have been developed for the production of rDNA products with strong positive ethnopharmacological correlation with the traditional knowledge as well as therapeutic potential, including bacteria, yeast, bird cells, insect cells, mammalian cells, etc. which possess specific platforms (Dossey 2010). Insects, since thousands of years, provided many valuable natural substances, including honey, silk, and other products (i.e. jelly, wax, etc.). Also, insect secretions and ground-up

M. Yusuf
Department of Chemistry, YMD College, M. D. University, Nuh, Haryana 122107, India
e-mail: yusuf1020@gmail.com

D. Kumar, C. Gong (eds.), *Trends in Insect Molecular Biology and Biotechnology*, https://doi.org/10.1007/978-3-319-61343-7_13

bodies have commonly been used as an important folklore medicine around the globe, for example, India, China, and European, African, and American countries. Literature published have described variable alluring accounts on insect-based folk medicine in India, Zaire, and Bolivian Amazon and other regions toward the use of insects and their secreted products as folk medicine for a huge range of treatments including allergy, anemia, arthritis, and bronchitis and some antivenoms (Dyck et al. 2003; Ratcliffe et al. 2011; Cherniack 2010; Ratcliffe et al. 2014).

Some studies have been published onto insect-derived natural products and their latent abilities in targeted drug development and treatment for various human diseases (Ratcliffe et al. 2011; Cherniack 2010). Among them, transgenic insect cells are known to be realized in cost-effective recombinant material production as a new expression system (Grzelak 1995; Kuwana et al. 2014). In this chapter transgenic insect-derived natural products and their potent applications are discussed through current researches.

13.2 Recombinant Silk Proteins

Human civilization has utilized insect silks since thousands of years because of their advantages and comfortness. Silks are protein materials produced by a wide range of insects and spider species for applications requiring high-performance fibers. The most common example is the use of reeled silkworm, *Bombyx mori*, to produce textiles which are used as a suture biomaterial for centuries, and also farmed silkworm silk in recent years, reprocessed into many forms such as films, gels, and sponges for biomedical applications. Despite the more promising and inherent abilities of insect-derived silk, spiders have not been domesticated for large scale or even industrial applications, since farming the spiders is not commercially viable due to their highly territorial and cannibalistic nature (Vepari and Kaplan 2007). There are chiefly two kinds of silk proteins which are derived, such as spiders and honeybees.

13.2.1 Spider Silk Proteins

Spider dragline silk proteins possess excellent mechano-physical strength and toughness, primarily composed of two related proteins that largely consist of repetitive sequences. Almost all spiders have proline-rich repetitive sequences rather than other types of proteins in majority. Nevertheless, due to excellent mechanical and biophysical properties, spider silks have been tailored as one of the most emerging research topics. The commonest silkworm, *Bombyx mori*, generates silk proteins in its silk glands and spins them as threads to build a cocoon in high yield. In general, each cocoon is made of a single thread more than 1000 m long, and about 3000 cocoons are needed to make a kilogram of silk. Silk is being a bulk product; it is believed that worldwide, 70,000 tons of silk are produced annually (Tomita 2011). There are around 34,000 species of spiders, all have a diversified tool kit for

production of mottled silks with divergent mechanical properties, and most of them make rather elaborate nests, traps, and cocoons using typically more than one type of silk-producing glands, spigots, and ducts (Scheibel 2004). However, the natural spiders cannot cover the current global needs, and therefore, the recombinant DNA technology exists to handle the situation for silk production on a super large scale, and one may reasonably assume the possibility of achieving the mass production of recombinant proteins in an approach similar to the desirable silk (Kuwana et al. 2014; Tomita 2011; Scheibel 2004).

Structurally, spider silks are natural polymers consist of three clarified domains (Fig. 13.1), for example, repetitive middle-core domain and non-repetitive N-terminal and C-terminal domains. Generally, silks differ in primary sequence, physical properties, and functions, for example, dragline silks used to build frames, radii, and lifelines are known for dazzling mechanical properties including elasticity, strength, and toughness (Gatesy et al. 2001; Tokareva et al. 2013). The most studied silk is dragline silk that shows remarked elasticity as well as strength. In a common way, *N. Clavipes*, the golden orb-spider produces dragline silk in the major ampullate gland. Also, it has been found that dragline silk is a protein complex,



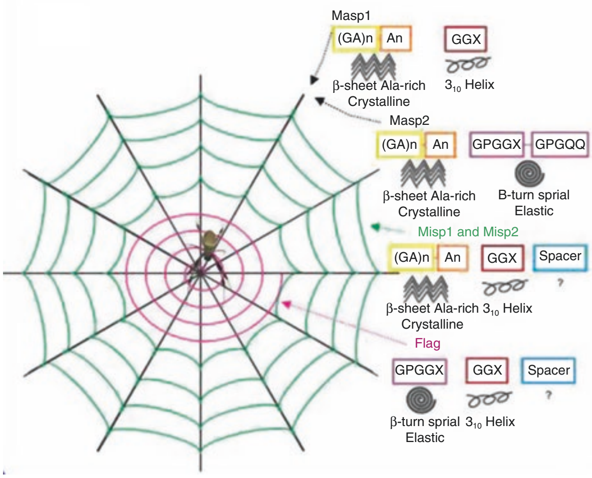


Fig. 13.1 (**a**) An adult female orb-weaver spider *Nephila clavipes* with her web. (**b**) Schematic overview of *N. clavipes* web composed of three different spider silk proteins and their structures ("spacer" region is unknown): (1) MaSp1 or MaSp2, major ampullate spidroin 1 or 2. (2) MiSp1 and 2, minor ampullate spidroin 1 and 2. (3) Flag, flagelliform protein. Adapted from Gatesy et al. (2001) with permission, Copyright John Wiley and Sons 2013

contains approximately 3500 amino acids, and is composed of mainly ampullate dragline silk protein 1 (MaSp1: found in periphery and fiber core) and major ampullate dragline silk protein 2 (MaSp2: form clusters and found in certain core areas). Subsequently, two basic sequences, crystalline (poly(A) or poly(GA)) and less crystalline (GGX or GPGXX) polypeptides, are alternatively placed to the large central core domains (MaSp1 and MaSp2), organized in a block copolymer-like arrangement. The main difference between MaSp1 and MaSp2 is the presence of proline residues accounting for 15% of the total amino acid content in MaSp2, while MaSp1 proline-free is reported (Gatesy et al. 2001; Tokareva et al. 2013; Hu et al. 2006). However, spiders have different ratios of MaSp1 and MaSp2. For example, the orb-weaver *Argiope aurantia* contains 41% MaSp1 and 59% MaSp2, and *N. clavipes* dragline silk contains 81% MaSp1 and 19% MaSp2 (Huemmerich et al. 2004; Brooks et al. 2005). Furthermore, secondary structures were assigned to poly(A)/(GA), GGX, and GPGXX motifs as β-sheet, 3_{10}-helix, and β-spiral, respectively. Consequently, Humenik et al. (2011) described the primary amino acid sequence, composition, and secondary structural elements (Humenik et al. 2011).

Nuclear polyhedrosis virus (BmNPV) was successfully introduced as a vector for transient expression system of recombinant proteins in *B. mori*. This system was focused on the expression of recombinant proteins which are found limited to one generation, and, therefore, viral infection must be conducted at every generation to allow the expression of proteins (Gosline et al. 1984; Maeda et al. 1985; Choudary et al. 1995). The synthesis of monomeric silk-like gene sequences as short single-stranded oligonucleotides (up to 100 bp) can be achieved by using commercial oligonucleotide synthesis or directly as polymerase chain reaction products from cDNA libraries. To the large-scale rDNA technology-based production, large repetitive sequences can be constructed by using concatemerization, step-by-step directional approach, and recursive ligation processes (Meyer and Chilkoti 2002; Higashiya et al. 2007). In order to understand the challenges and needs associated with spider silks, a common primary structural pattern is comprised of a large central core of repetitive protein domains flanked by non-repetitive domains. Systematic approach was deliberated toward transient unicellular organisms, such as bacteria and yeast, as host systems for recombinant silk production. The industrial-scale production of spider silk proteins was established suitably and reported by using a rod-shaped, Gram-negative bacterium *E. coli* as a remarked host system (Fahnestock and Irwin 1997; Wang et al. 2006; Lewis 2006; Rabotyagova et al. 2010; Teulé et al. 2012a; An et al. 2012).

The laboratory scale production of recombinant spider silk would initiate a new generation of ecological materials and is recognized as a promising tool with broad usability in medical sector (Tomita 2011; Gatesy et al. 2001; Teulé et al. 2012a). In addition, due to brilliant performance, spider silks can be further used in artificial ligaments and tendons for durable implantation (Ding et al. 2014). High strength and flexible performance fibers built from recombinant spider silks can be employed in several technical and industrial applications. In addition to specialty ropes and fishing nets, spider silk can be utilized for making sporting goods, textiles, parachutes, and frivolous constructions for airplanes (Hu et al. 2012). In desired

Table 13.1 Potential use of silk biopolymers in medicine (Ratcliffe et al. 2011; Tomita 2011; Teulé et al. 2012a; An et al. 2012; Ding et al. 2014; Hu et al. 2012; Zhang et al. 2013; Demain and Vaishnav 2009)

Type of silk proteins	Potent use
Nanoparticles	Delivery of drugs to cancer cells
B. mori porous materials	For repair of cartilage, bone, ligaments, tendons, vascular tissue, nerves, and corneas and as wound dressings
Silk-heparin support	Vascular tissue growth application
Copolymer blocks	Transfection of target cancer cells
Small, globular units with tumor-homing peptides (THP)	Improved tumor cell-specific transfection
Ionic complexes of nano-scaled silk with THP	Further improved tumor cell-specific transfection
Silk hydrogels	Treatment of breast cancer
Antibiotic-loaded silk hydrogels	Prevention and treatment of infection
Electrically stimulated silk films	Enhancement of neural growth
Vitamin E-loaded silk nanofibrous mats	Skin tissue regeneration
Silk protein matrices	Thermostabilization of vaccines

proportions silk proteins are massively generated and gathered high repute applications in genetic engineering to meet the copious demands for industrial espionage and also in rapid progress are being made in the development of silk for use in medicine (Table 13.1) (Ratcliffe et al. 2011; Zhang et al. 2013). Hence, spider silks are also favorable as commence biomaterials (Demain and Vaishnav 2009; Omenetto and Kaplan 2010). Furthermore, with a broader screening of other naturally fibrous proteins, insect silks can be grouped into more than 23 independently evolved lineages with different secondary structures such as cross-beta sheets, coiled coils, or polyglycine II structures that may be utilized to the next generation through various novel applied sectors.

Even so, in a consequent study, silkworms transformed with chimeric silkworm/spider silk genes spin composite silk fibers that were reported with improved mechanical properties. In this study, engineered transgenic silkworms were subjected to express the synthetic $A2S8_{14}$ spider silk gene in an effort to produce composite fibers consisting, at least in part, of the synthetic spider silk protein, and results showed that transgenic silkworms encoding synthetic spider silk proteins can, indeed, spin composite silk fibers with improved mechanical properties, relative to the fibers produced by the parental animals tested (Teulé et al. 2012b).

13.2.2 Honeybee Silk Proteins

Similar to spider silks (i.e., *Bombyx mori*), honeybees also secrete four different types of small coiled-coil proteins with a molecular weight of about 30 kDa and were found to be stable in water. These proteins are non-repetitive and rich in alanine residues chiefly. The ingenuity of science continues to amaze with the

transient honeybee silk recently produced as biomaterials for the transport and drug delivery and also in tissue engineering. Nevertheless, the particular interest is being under consideration about the silk from bees, ants, and hornets of Hymenoptera type (Sutherland et al. 2010). The hymenopteran silk has the molecular structure of type α-helical proteins assembled into a tetrameric coiled conformation and also fundamental design to the β-sheet plesiomorphic crystallites that dominate the silkworm cocoon and spider dragline silks reported (Atkins 1967; Sutherland et al. 2011). The spatial and sequential arrangement of repetitive units/amino acids arises within the proteins, and these features promote self-assembly and formation of structural hierarchy in consequence to material-related functional roles (Sutherland et al. 2011; Poole et al. 2013). For example, recombinantly accessed Asiatic honeybee silk was obtained from *Apis cerana* (Shi et al. 2008). A flexible and solvent-stable fiber production was described by Poole et al. (2013). In this method after concentrated recombinant honeybee protein solutions were extruded into a methanol bath, dried, drawn in aqueous methanol, then covalently cross-linked using dry heat and solvent-stable and flexible fibers were fabricated. In another study, biomimetic spinning system was employed for recombinant production and purification of the four full-length unmodified honeybee silk proteins in *E. coli* at substantial yields of 0.2–2.5 g/L under the correct conditions the recombinant proteins self-assembled to reproduce the native coiled-coil structure (Weisman et al. 2010).

In the present scenario, naturally originated biomimetic biomaterials are much attentive and noteworthy targets for tissue engineering and other biomedical applications. In a study, honeybee silk membranes were described to have bright exploration toward their applicability for tissue engineering and found capabilities such as biodegradation and considerable mechanical and biological properties (Kumar et al. 2016). In addition, honeybee recombinant silk proteins have recently gained interest as materials for bioengineering and nanomedicine as they possess several features with significant functionality, biocompatibility, and degradability (Corchero et al. 2014).

13.3 Collagen Proteins

Collagen is one of the most abundant proteins found in tissues of animals, including tendons, ligaments, and skin, and is ubiquitous throughout the animal kingdom, where it comprises some 28 diverse molecules that form the extracellular matrix within organisms, and many of those recently discovered are present in tissues in small. In general, collagen consists of different chain compositions depending on the specific types and is composed of three left-handed helices disheveled to form a right-handed triple helix with (GPX)*n*, as commonest amino acids, in which X is any amino acid other than proline, glycine, or hydroxyproline (Sutherland et al. 2013; Browning et al. 2012). Although recombinant collagen proteins could be produced by bacterial and insect cells as standard expression systems, they facilitate in

opening the new opportunistic door to manufacture. For example, triple-helical procollagen from recombinant type-III procollagen was produced in a study by co-expressing together with mammalian P4H in the yeast cell, *Pichia pastoris* (Vuorela et al. 1997). Another study was carried out on insect cells as expression carrier, and as a consequence it was observed that insect cells possess a very low level of endogenous P4H activity; co-transfection of such cells with a combination of baculovirus vectors encoding human type-III procollagen and both α- and β-subunits of P4H yielded up to 60 μg/mL cellular procollagen (Lamberg et al. 1996). However, triple-helical collagen molecules showed to have an ability to assemble into favorable supramolecular structures that form the basis of commercial uses of collagen in real industrial sectors, such as food, tissue engineering, and medical applications (Sutherland et al. 2013; Lamberg et al. 1996).

13.4 Cantharidin

Robiquet, a French chemist, in 1810 isolated cantharidin (Fig. 13.2), firstly. It is an odorless and colorless monoterpene, secreted by about 1500 different species of blister beetles, including broadly known sources Spanish fly, in genus *Epicauta* and in species *Lytta vesicatoria*, and has an important role in the ecology of different kinds of insects that use or produce it as a defense to preserve their eggs from predators (Ratcliffe et al. 2014; Wang 1989).

Despite the development of less toxic analogues, there are significant researches onto naturally derived products. However, cantharidin is found to cause adverse effects, included in a list of "problem drugs." Nevertheless, cantharidin is found to have good antimicrobial, antileishmanial, and anticancer abilities and can be safely and effectively used to treat some benign skin lesions like warts and molluscum under cautious supervision (Moed et al. 2001; Ghaffarifar 2010). Consequently, an important orally inhalable therapeutic use of cantharidin has tackled the problems of its toxicity, short half-life period, and insolubility in circulation by using solid lipid nanoparticles as drug carriers (Dang and Zhu 2013). Another chemical genomics study has been performed by Lissina et al. (2011) and observed that cantharidin is found to be an effective gene probe of transcriptional regulation for CRG1 gene (Lissina et al. 2011).

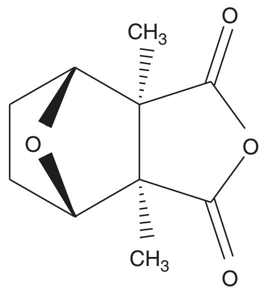


Fig. 13.2 Chemical structure of cantharidin

13.5 Antimicrobial Peptides (AMPs)

Undoubtedly, insects are one of the best repertoire sources for antimicrobial peptides (AMPs), and their potent antimicrobial activity to address the threat of multidrug-resistant pathogens, in vitro and in vivo, has encouraged their development as alternatives to conventional antibiotics (Dossey 2010; Ratcliffe et al. 2011). Chemically insect-derived AMPs are mainly cationic (although anionic forms do exist to a significant extent), facilitate their binding through electrostatic force to negatively charged bacterial and tumor cell walls, and are also amphipathic in their folded state with hydrophilic and hydrophobic regions mediating their solubility in phospholipid cell membranes. These interactions of the AMPs resulted disruptive capabilities (Ratcliffe et al. 2014; Ntwasa 2012).

Insect AMPs can be classified on the basis of their structure or function and can be categorized into three groups, as under:

13.5.1 Linear α-Helical AMPs

Linear *α*-helical AMPs are present in a wide range of insect orders, including coleopterans, dipterans, and lepidopterans, for example, cecropins, moricin, sarcotoxin, and melittin, out of which cecropin is the firstly discovered AMP produced by larvae of the giant silk moth *Hyalophora cecropia*, prototype *α*-helical linear AMP, active against Gram-negative bacteria such as *E. coli*. In general, cecropins contain a tryptophan residue at or near the N-terminus, a long N-terminal amphiphilic α-helix, a shorter and more hydrophobic α-helix at the C-terminus, and an amidated C-terminal residue. Furthermore, cecropins are found to be active against Gram-positive and Gram-negative bacteria, viruses, protozoans, fungi, nematodes, and tumor cells (Ratcliffe et al. 2014; Gaspar et al. 2013). Consequently, some new additional cecropin-like peptides such as enbocin, sarcotoxins, and hyphancin with selective antimicrobial potential against both Gram-positive and Gram-negative bacteria (Ratcliffe et al. 2011, 2014; Gaspar et al. 2013; Mylonakis et al. 2016).

13.5.2 Linear Proline or Glycine-Rich AMPs

Linear proline or glycine-rich AMPs, is another class that contains characteristic multiple proline residues confers targeted antimicrobial specificity. Several orders have been found to be enriched with proline or glycine, for example, Diptera (drosocin and metchnikowin), Hymenoptera (apidaecins, abaecins, and formaecins), Hemiptera (pyrrhocoricin and metalnikowins), and Lepidoptera (lebocins) (Ratcliffe et al. 2014; Gaspar et al. 2013; Mylonakis et al. 2016). Moreover, this class can be further divided into two subclasses such as short chain (contains about 20 residues) and long chain (contains more than 20 residues), out of which the former subclass showed more potent activity toward Gram-negative bacteria, while the latter one observed superior activity against Gram-positive bacteria and fungi (Mylonakis et al. 2016; Rahnamaeian and Vilcinskas 2012).

13.5.3 Cysteine-Stabilized AMPs

Furthermore, a class pertaining insect-derived antimicrobial peptides is cysteine-stabilized AMPs. In this class, small cationic peptides with 33–46 amino acids have a predominantly β-sheet globular structure and are stabilized by cysteine residues through intramolecular disulfide bridges. The most studied examples for this class are defensins, which represent the prototype for major structural class of insect-derived AMPs (Ratcliffe et al. 2014; Mylonakis et al. 2016). Defensins and defensin-like compounds (i.e., gallerimycin, heliomycin, sapecins, drosomycin, spodoptericin, and phormicins) are isolated from many insect orders such as, Hemiptera, Odonata, Coleoptera, Diptera, Hymenoptera, and Lepidoptera. Many of these compounds have shown not only potent activity against Gram-positive bacteria as well as fungi but also considerable antiparasitic capability (Mylonakis et al. 2016; Poppel et al. 2015).

13.6 Other Products

From ancient times around the globe, honey has been obtained from bees and used as important folklore medication for several ailments and diseases. Surprisingly, despite the success of insects in terms of numbers and diversity, recently, the potent activity of honey has been extensively studied. Interestingly, honey was found to have brilliant antimicrobial performance against several antibiotic-resistant human pathogens (Ratcliffe et al. 2011; Cherniack 2010). The presence of large number of bioactive phenolic compounds (Table 13.2) in honey has gaining popularity to the use of honey in clinical practices and trials and is evidently found to be a great potential, for example, wound care anticancer, antimicrobial, antispasmodic, and other beneficial abilities (Ratcliffe et al. 2014; Mylonakis et al. 2016).

Bee venoms include a large range of practically unexplored compounds awaiting discovery and development into the future medicines, for example, some ant and parasitoid wasp venoms may contain even more than 75 more different components. Nevertheless, bee venom therapy also has been used in folk medicine for many thousands of years to treat several ailments from arthritis, skin diseases, multiple sclerosis, rheumatism, cancer, infections, and pain (Ratcliffe et al. 2014; Mylonakis et al. 2016; Al-Waili et al. 2011).

Table 13.2 Bioactive phenols present in honey (Ratcliffe et al. 2014; Mylonakis et al. 2016)

Class of phenolic compounds	Typical examples
Phenolic acids	Caffeic acid
Coumarins	Coumarin
Tannins	Ellagic acid
Flavonols	Quercetin, kaempferol, galangin, fisetin, myricetin, etc.
Flavanones	Hesperidin
Flavones	Apigenin, acacetin, chrysin, luteolin, genkwanin, wogonin, and tricetin

13.7 Conclusion and Future Outlook

In the present work, insect-derived natural products based on rDNA technology are discussed which are of great interest in recent era, such as proteins/peptides (silks, collagen, cantharidin, AMPs) and others (honey, venom, etc.). Spider dragline and honeybee silks having significant functionality, biocompatibility, and degradability possess high tensile strength, flexible performance, and excellent mechanical properties suitable for significant biomedical applications. Collagen and cantharidin proteins are also found applicable toward food, tissue, and medical engineering. Furthermore, antimicrobial peptides (AMPs) with potent antimicrobial activity toward multidrug-resistant pathogens, in vitro and in vivo, have encouraged their development as alternatives to conventional antibiotics. The significant step toward the progression in transient organisms surely suggests new directions to emulate in the pursuit of new high-performance, multifunctional materials generated with green platforms that integrate with living systems that could be used for various applications, including cosmetic, diagnostic, animal therapeutic, and human therapeutic uses. Thus, the spectrum of tunable functional materials is expected to grow exponentially in the next years and fulfill the demands of new targeted, sustained drug delivery platforms and functional scaffolds using recombinant DNA technology.

References

Al-Waili NS, Salom K, Al-Ghamdi AA (2011) Honey for wound healing, ulcers, and burns; data supporting its use in clinical practice. Sci World J 11:766–787

An B, Jenkins JE, Sampath S et al (2012) Reproducing natural spider silks' copolymer behavior in synthetic silk mimics. Biomacromolecules 13:3938–3948

Atkins EDT (1967) A four-strand coiled-coil model for some insect fibrous proteins. J Mol Biol 24:139–141

Brooks AE, Steinkraus HB, Nelson SR et al (2005) An investigation of the divergence of major ampullate silk fibers from *Nephila clavipes* and *Argiope aurantia*. Biomacromolecules 6:3095–3099

Browning MB, Dempsey D, Guiza V et al (2012) Multilayer vascular grafts based on collagen-mimetic proteins. Acta Biomater 8:1010–1021

Cherniack EP (2010) Bugs as drugs, part 1: insects. The "new" alternative medicine for the 21st century? Altern Med Rev 15(2):124–135

Choudary PV, Kamita SG, Maeda S (1995) Expression of foreign genes in *Bombyx mori* larvae using baculovirus vectors. Methods Mol Biol 39:243–264

Corchero JL, Vázquez E, García-Fruitós E et al (2014) Recombinant protein materials for bioengineering and nanomedicine. Nanomedicine 9(18):2817–2828

Dang YJ, Zhu CY (2013) Oral bioavailability of cantharidin-loaded solid lipid nanoparticles. BMC Chin Med 8:1. doi:10.1186/1749-8546-8-1

Demain AL, Vaishnav P (2009) Production of recombinant proteins by microbes and higher organisms. Biotechnol Adv 27(3):297–306

Ding D, Guerette PA, Hoon S et al (2014) Biomimetic production of silk-like recombinant squid sucker ring teeth proteins. Biomacromolecules 15(9):3278–3289

Dossey AT (2010) Insects and their chemical weaponry: new potential for drug discovery. Nat Prod Rep 27(12):1737–1757

Dyck MK, Lacroix D, Pothier F et al (2003) Making recombinant proteins in animals different systems, different applications. Trends Biotechnol 21:394–399

Fahnestock SR, Irwin SL (1997) Synthetic spider dragline silk proteins and their production in Escherichia coli. Appl Microbiol Biotechnol 47:23–32

Gaspar D, Veiga AS, Castanho MARB (2013) From antimicrobial to anticancer peptides: a review. Front Microbiol 4:294. doi:10.3389/fmicb.2013.00294

Gatesy J, Hayashi C, Motriuk D et al (2001) Extreme diversity, conservation, and convergence of spider silk fibroin sequences. Science 291(5513):2603–2605

Ghaffarifar F (2010) Leishmania major: In vitro and in vivo anti-leishmanial effect of cantharidin. Exp Parasitol 126(2):126–129

Gosline JM, Denny MW, DeMont ME (1984) Spider silk as rubber. Nature 309:551–552

Grzelak K (1995) Control of expression of silk protein genes. Comp Biochem Physiol B Biochem Mol Biol 110:671–681

Higashiya S, Topilina NI, Ngo SC et al (2007) Design and preparation of b-sheet forming repetitive and block-copolymerized polypeptides. Biomacromolecules 8:1487–1497

Hu X, Vasanthavada K, Kohler K et al (2006) Molecular mechanisms of spider silk. Cell Mol Life Sci 63:1986–1999

Hu X, Cebe P, Weiss AS et al (2012) Protein-based composite materials. Mater Today 15(5):208–215

Huemmerich D, Helsen CW, Quedzuweit S et al (2004) Primary structure elements of spider dragline silks and their contribution to protein solubility. Biochemist 43:13604–13612

Humenik M, Scheibel T, Smith A (2011) Spider silk understanding the structure-function relationship of a natural fiber. Prog Mol Biol Transl Sci 103:131–185

Kumar M, Jain D, Bhardwaj N et al (2016) Native honeybee silk membrane: a potential matrix for tissue engineering and regenerative medicine. RSC Adv 6(59):54394–54403

Kuwana Y, Sezutsu H, Nakajima KI et al (2014) High-toughness silk produced by a transgenic silkworm expressing spider (*Araneus ventricosus*) dragline silk protein. PloS One 9(8):e105325, 1-11

Lamberg A, Helaakoski T, Myllyharju J et al (1996) Characterization of human type III collagen expressed in a baculovirus system production of a protein with a stable triple helix requires coexpression with the two types of recombinant prolyl 4-hydroxylase subunit. J Biol Chem 271(20):11988–11995

Lewis RV (2006) Spider silk: ancient ideas for new biomaterials. Chem Rev 106:3762–3774

Lissina E, Young B, Urbanus ML et al (2011) A systems biology approach reveals the role of a novel methyltransferase in response to chemical stress and lipid homeostasis. PLoS Genet 7(10):e1002332

Maeda S, Kawai T, Obinata M et al (1985) Production of human alpha-interferon in silkworm using a baculovirus vector. Nature 315:592–594

Meyer DE, Chilkoti A (2002) Genetically encoded synthesis of protein-based polymers with precisely specified molecular weight and sequence by recursive directional ligation: examples from the elastin-like polypeptide system. Biomacromolecules 3:357–367

Moed L, Shwayder TA, Chang MW (2001) Cantharidin revisited: a blistering defense of an ancient medicine. Arch Dermatol 137(10):1357–1360

Mylonakis E, Podsiadlowski L, Muhammed M, Vilcinskas A (2016) Diversity, evolution and medical applications of insect antimicrobial peptides. Philos Trans R Soc B 371:20150290. doi:10.1098/rstb.2015.0290

Ntwasa M (2012) Cationic peptide interactions with biological macromolecules. In: Abdelmohsen K (ed) Binding Protein. InTech, Croatia, pp 139–164

Omenetto FG, Kaplan DL (2010) New opportunities for an ancient material. Science 329(5991):528–531

Poole J, Church JS, Woodhead AL et al (2013) Continuous production of flexible fibers from transgenically produced honeybee silk proteins. Macromol Biosci 13(10):1321–1326

Poppel AK, Vogel H, Wiesner J, Vilcinskas A (2015) Antimicrobial peptides expressed in medicinal maggots of the blow fly *Lucilia sericata* show combinatorial activity against bacteria. Antimicrob Agents Chemother 59:2508–2514

Rabotyagova OS, Cebe P, Kaplan DL (2010) Role of polyalanine domains in β-sheet formation in spider silk block copolymers. Macromol Biosci 10:9–59
Rahnamaeian M, Vilcinskas A (2012) Defense gene expression is potentiated in transgenic barley expressing antifungal peptide Metchnikowin throughout powdery mildew challenge. J Plant Res 125:115–124
Ratcliffe NA, Mello CB, Garcia ES et al (2011) Insect natural products and processes: new treatments for human disease. Insect Biochem Mol Biol 41(10):747–769
Ratcliffe N, Azambuja P, Mello CB (2014) Recent advances in developing insect natural products as potential modern day medicines. Evid Based Complement Alternat Med 2014:1–21
Scheibel T (2004) Spider silks: recombinant synthesis, assembly, spinning, and engineering of synthetic proteins. Microb Cell Fact 3:1–14
Shi J, Lua S, Du N, Liu X, Song J (2008) Identification, recombinant production and structural characterization of four silk proteins from the Asiatic honeybee *Apis cerana*. Biomaterials 29:2820–2828
Sutherland TD, Young J, Weisman S et al (2010) Insect silk: one name, many materials. Annu Rev Entomol 55:171–188
Sutherland TD, Church JS, Hu X et al (2011) Single honeybee silk protein mimics properties of multi-protein silk. PLoS One 6(2):e16489
Sutherland TD, Peng YY, Trueman HE et al (2013) A new class of animal collagen masquerading as an insect silk. Sci Rep 3:2864
Teulé F, Addison B, Cooper AR et al (2012a) Combining flagelliform and dragline spider silk motifs to produce tunable synthetic biopolymer fibers. Biopolymers 97:418–431
Teulé F, Miao YG, Sohn BH et al (2012b) Silkworms transformed with chimeric silkworm/spider silk genes spin composite silk fibers with improved mechanical properties. Proc Natl Acad Sci 109(3):923–928
Tokareva O, Michalczechen-Lacerda VA, Rech EL et al (2013) Recombinant DNA production of spider silk proteins. Microb Biotechnol 6(6):651–663
Tomita M (2011) Transgenic silkworms that weave recombinant proteins into silk cocoons. Biotechnol Lett 33(4):645–654
Vepari C, Kaplan DL (2007) Silk as a biomaterial. Prog Polym Sci 32:991–1007
Vuorela A, Myllyharju J, Nissi R et al (1997) Assembly of human prolyl 4-hydroxylase and type III collagen in the yeast Pichia pastoris: formation of a stable enzyme tetramer requires coexpression with collagen and assembly of a stable collagen requires coexpression with prolyl 4-hydroxylase. EMBO J 16(22):6702–6712
Wang GS (1989) Medical uses of mylabris in ancient China and recent studies. J Ethnopharmacol 26(2):147–162
Wang C, Patwardhan SV, Belton DJ et al (2006) Novel nanocomposites from spider silk-silica fusion (chimeric) proteins. Proc Natl Acad Sci U S A 103:9428–9433
Weisman S, Haritos VS, Church JS et al (2010) Honeybee silk: recombinant protein production, assembly and fiber spinning. Biomaterials 31(9):2695–2700
Zhang J, Pritchard E, Hu X et al (2013) Stabilization of vaccines and antibiotics in silk and eliminating the cold chain. Proc Natl Acad Sci U S A 109(30):11981–11986

Structure, Regulation, and Potential Applications of Insect Chitin-Metabolizing Enzymes

14

Manish Kumar, V. Vivekanand, and Nidhi Pareek

Abstract

Chitin is a vital component of insect exoskeleton and peritrophic matrix and because of this reason a potential target for insecticidal agents. Chitin-metabolizing enzymes, viz., chitin synthases and chitinases, belong to the glycoside hydrolase superfamily (GH18). Chitin synthases are involved in deposition of new cuticle during molting and also ideal for development of insecticidal agents. Chitinases are considered as an essential enzyme for insect growth and development being involved in molting and various other physiological processes, i.e., cuticle turnover, regulation of abdominal contraction and wing expansion, digestion, immunity, and natural defense. Chitinases possess multi-domain architecture, i.e., chitin-binding domain, Ser-/Thr-rich linker domains, catalytic domains, fibronectin, and mucin-like domains. Knockdown of both the enzymes resulted into irregularities in metamorphosis. Diverse group of chitinase-like proteins have also been detected in insect species that possess chitin-binding domains but do not exhibit catalytic activity. Development of chitinases as defensive agents against chitin-bearing insect pests and pathogens will generate new knowledge and innovative processes for biocontrol advancements.

M. Kumar • N. Pareek (✉)
Department of Microbiology, School of Life Sciences, Central University of Rajasthan, Bandarsindri, Kishangarh, Ajmer 305801, Rajasthan, India
e-mail: nidhipareek@curaj.ac.in

V. Vivekanand
Centre for Energy and Environment, Malaviya National Institute of Technology, Jaipur 302017, Rajasthan, India

D. Kumar, C. Gong (eds.), *Trends in Insect Molecular Biology and Biotechnology*, https://doi.org/10.1007/978-3-319-61343-7_14

14.1 Introduction

Chitin is the second most abundant insoluble structural polymer in nature following cellulose and is composed of linear chains of β-1,4-linked *N*-acetyl-D-glucosamine (GlcNAc) residues. It is the essential component of fungal cell wall, house dust mites, exoskeletons of crabs, shrimp and insects, parasitic nematodes, and digestive tracts of many insects (Pillai et al. 2009). In insects (Arthropoda), chitin is mainly found in the exoskeleton and peritrophic matrix (PM). The PM is a structure in the insect's gut, which consists mainly of proteins and glycosaminoglycans embedded in a chitinous matrix that protect insects against the mechanical damage and pathogens (Chapman 1998). The development of the cuticle which is the complex exoskeleton with diverse functions is the key factor responsible for the evolutionary success of insects (Wittkopp and Beldade 2009). Apart from these two structures, chitin has also been identified as a component of the salivary glands, trachea, tracheiolas, ovaries, dermal glands, eggs, and eggshell in insects. The growth and development in insects are regulated by the metabolism of chitin in the cuticle and the PM. In insects, the rigidity of the cuticle although provides physical support and protection, it also results in the restriction of growth and development (Tetreau et al. 2015). This restriction is compensated by molting, i.e., a complex and multistep cascade including a series of enzymes and cofactors. Therefore, these chitin-related enzymes and proteins play a key role in the insect's metabolism and help in their growth, development, and survival. On the basis of the function, the enzymes involved in chitin metabolism can be divided into three major categories, i.e., synthetic (chitin synthases), modifying (chitin deacetylases, to enzymatically alter chitin by deacetylation), and the degradative enzymes (chitinases and *N*-acetylglucosaminidases, to degrade chitin by hydrolytic process) (Chen 1987; Merzendorfer and Zimoch 2003). Among these, chitin synthases and chitinases are considered as the key enzymes and widely studied. Chitinases can be further divided into endochitinases (EC 3.2.1.14) and exochitinases (EC 3.2.1.52) on the basis of their mode of action. The former cleave within the chitin polymer to release long-chain chitin oligosaccharides, and the latter releases short-chain oligomers.

14.2 Chitin Synthases

Chitin synthases (CHS) belongs to family 2 of glycosyltransferases (GT2) in the carbohydrate-active enzyme (www.cazy.org) database (Lombard et al. 2014; Coutinho et al. 2003), which synthesize polymeric chitin. The GT2 also includes cellulose synthase (EC 2.4.1.34), hyaluronan synthase (EC 2.4.1.212), etc. CHS catalyzes the formation of different forms of chitin, i.e., α-form (the chains are in antiparallel orientation), β-form (the chains are arranged in parallel manner), and γ-form (sets of two parallel strands alternate with a single antiparallel strand) (Merzendorfer 2006; Kaya et al. 2013). The catalytic mechanism of CHS is still not well known. From research findings, it is clear that CHS is processive enzyme that bind to the polymer and add single GlcNAc to the nonreducing end. CHS reaction

requires UDP-*N*-acetylglucosamine as the substrate and divalent metal cations like Mg^{2+} or Mn^{2+} (Merzendorfer and Zimoch 2003; Saxena et al. 1995; Yeager and Finney 2004).

14.2.1 Structure and Catalytic Mechanism

Modular structure of CHS has been predicted and studied by various researchers. According to the modular structure given by Arakane et al. (2004), CHS are large membrane-integrated enzymes with various domains that are important for subcellular localization and activation. Like cellulose synthases, CHS has multiple transmembrane (TM) domains work as the transport channel for chitin deposition in the outer membrane (Morgan et al. 2013). CHS has three unique domains, *N*-terminal moderately conserved sequence domain, highly conserved catalytic domain, and the *C*-terminal module with multiple transmembrane segments. The catalytic domain contains several stretches of highly conserved amino acid sequences. There are different patterns of transmembrane segments of the *N*-terminal domain in different species of insects. In spite of domains, CHS also possesses several conserved motifs like EDR, QRRRW, and WGTRE (Morgan et al. 2013; Muthukrishnan et al. 2016; Moussian et al. 2005) (Fig. 14.1) in which EDR and QRRRW are closed to the active site (Merzendorfer 2006). The insect CHS has molecular mass in the range of 160–180 kDa and exhibit a slightly acidic isoelectric point between 6.1 and 6.7 (Merzendorfer and Zimoch 2003). On the basis of available crystal structure of a bacterial cellulose synthase, Dorfmueller et al. (2014) reported the chitooligosaccharide synthase model encoded by the bacterial *NodC* gene. In this study they proposed about the membrane spanning region of NodC protein and traversing of the lipid bilayer in three different orders, i.e., outside to inside, inside to outside, and outside to inside. On the basis of crystal structure of eight GT2 enzymes,

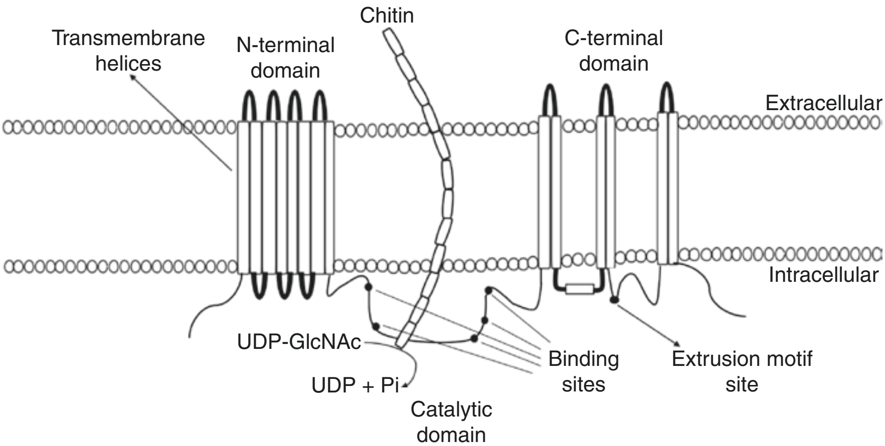


Fig. 14.1 Hypothetical model of the tripartite domain organization of the insect chitin synthase

Muthukrishnan et al. (2016) modeled an insect synthase and reported the presence of a long tunnel at the active site that is a remarkable difference between the NodC structure and that of the cellulose synthases and CHS. This long tunnel accommodates sugars of elongation chains of cellulose and chitin, whereas the NodC enzyme has a closed pocket which can accommodate only a pentasaccharide chain. The study also indicated that six transmembrane helices (TMH) are involved in the formation of narrow channel. The helix of the TMS region controls the entry of the chitin-conducting channel as it is attached to the cytosolic side of the membrane (Van Leeuwen et al. 2012; Demaeght et al. 2014). Muthukrishnan et al. (2012) have predicted the structural model of the tripartite domain organization of *Drosophila* DmCHS1. According to the model (Fig. 14.1), *N*-terminal domain contains eight TMH which vary from 7 to 10 among different insect species. The domain facing toward the cytoplasm is the middle catalytic domain which contains the catalytic sites like nucleotide-binding sites, donor saccharide-binding site, acceptor saccharide-binding site, and the product-binding site, and the domain C contains (5 + 2) conserved TMH. This model suggested that polymer synthesis occurs in the cytosol and further the chitin chain is translocated across the membrane by the help of five TMS clusters and the extrusion motif SWGTR. In the model, one helix appeared not to span the membrane but is attached to the cytosolic side of the plasma membrane assist in predicting the intracellular orientation of the *C*-terminus.

14.2.2 Chitin Biosynthesis

CHS plays a leading role in the biosynthesis of chitin. CHS requires UDP-*N*-acetylglucosamine (UDP-GlcNAc) as the activated sugar donor in the formation of the chitin in insects (Merzendorfer 2011; Moussian 2008). The biosynthetic pathway of chitin synthesis in insects starts with trehalose and ends with the chitin (Cohen 1987; Tharanathan and Kittur 2003). The major site for the chitin biosynthesis in insects is the epidermis and the midgut (Tellam et al. 2000). The epidermal cells cause the deposition of new cuticle during molting, and the midgut cells are related to the PM formation during feeding (Reynolds 1987; Zimoch et al. 2005). This biosynthesis can be grouped into three major steps. The first step deals with the formation of polymer in which enzymes' catalytic domain facing toward the cytoplasmic sites plays a crucial role. In the second step of biosynthesis, the nascent polymer translocated across the membrane followed by the third step where the process completes by the formation of crystalline microfibrils (Merzendorfer and Zimoch 2003; Muthukrishnan et al. 2012). These microfibrils further combine with other sugars and proteins to develop the insect's cuticle and PM. There are three key enzymes that act as the rate-limiting factors in the chitin biosynthesis pathway, i.e., glutamine-fructose-6-P aminotransferase (EC 2.6.1.16), UDP-GlcNAc pyrophosphorylase (EC 2.7.7.23), and chitin synthase (EC 2.4.1.16). Chitin biosynthesis pathway is published and well discussed in the book chapter by Kramer and Muthukrishnan (2009). In the biosynthesis pathway (Fig. 14.2), trehalose is the extracellular source of sugar and acted upon by

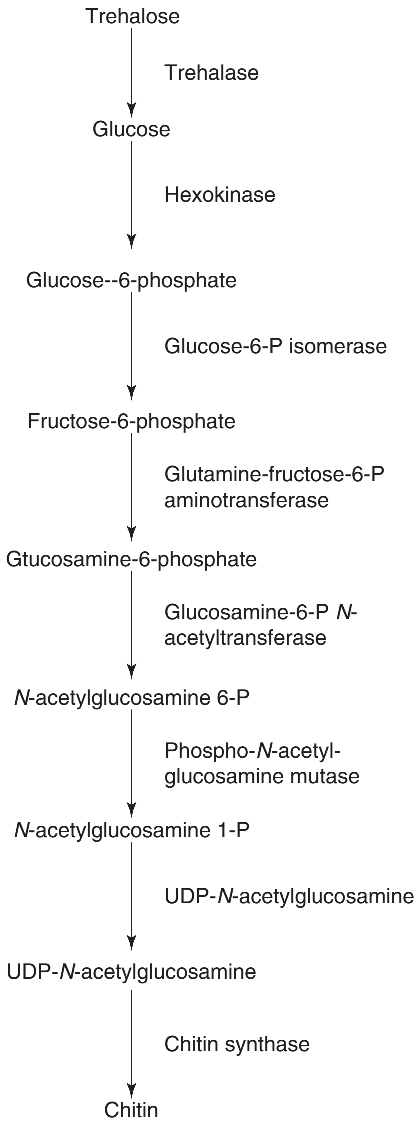


Fig. 14.2 Chitin biosynthesis pathway in insects

trehalase which is present in the insect. Further, hexokinase and glucose-6-P isomerase convert glucose to fructose-6-P. Formation of GlcNAc phosphate requires amination, acetyl transfer, and isomerization. The substrate for chitin synthase, UDP-GlcNAc, is formed from GlcNAc1-P by the action of UDP-GlcNAc pyrophosphorylase. Lastly, chitin synthase converts UDP-GlcNAc into chitin to complete the synthesis.

Dorfmueller et al. (2014) studied the structure and reaction mechanism of CHS by using the bacterial glycosyltransferase NodC as the model system. They deduced

a reaction mechanism for processive chitooligosaccharide synthesis (Dorfmueller et al. 2014; Stern and Jedrzejas 2008). In this mechanism, the 1-hydroxyl group of the donor substrate, UDP-GlcNAc is transferred onto the nonreducing end of the growing acceptor oligosaccharide. The acceptor GlcNAc sits in the A site with the 4-hydroxyl group pointing toward the UDP-GlcNAc-binding site. This acceptor sugar is activated by the catalytic base, i.e., Asp-241 in *Sm*NodC, and perform a nucleophilic attack on the anomeric carbon of UDP-GlcNAc, generating a β-(1,4)-glycoside. After the completion of the transfer, UDP leaves the active site. In the first synthesis step, the two terminal sugars (donor and A) of the growing chain would rotate while moving to the next binding site (A and B). During further elongation, the A sugar would only rotate every second synthesis step (blue sugar compared with green sugar). All sugars moving into the C/D subsites would remain in a fixed orientation. This orientation and translocation enable the newly added nonreducing sugar to be in the same acceptor position as the previous one (Fig. 14.3) (Dorfmueller et al. 2014).

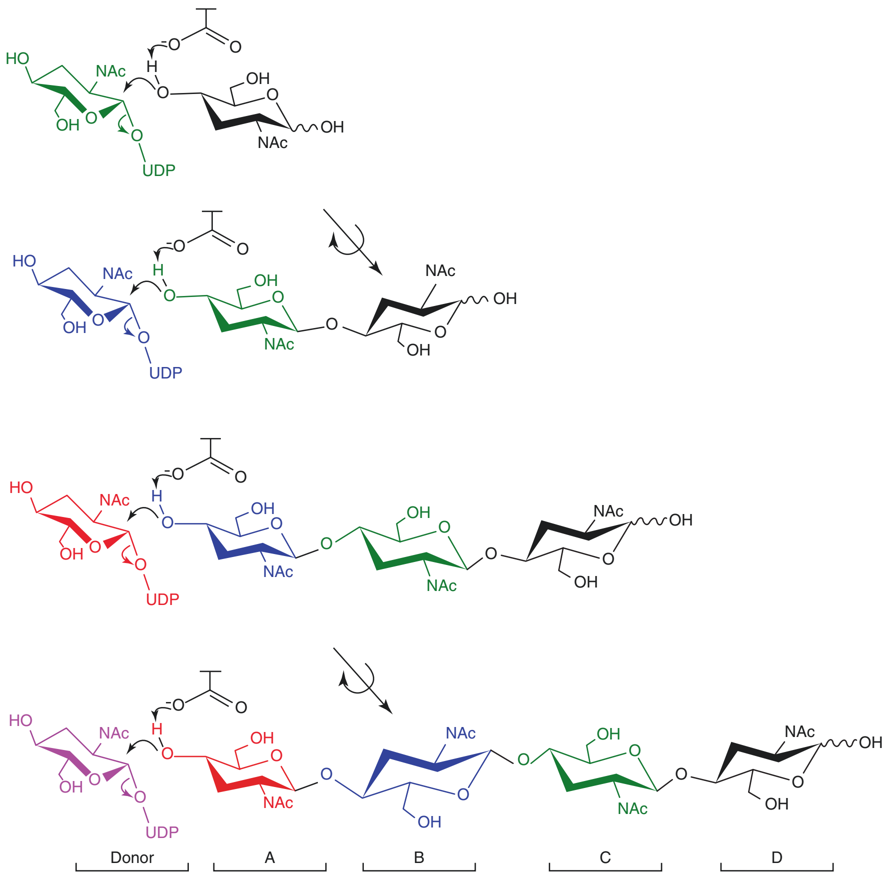


Fig. 14.3 Reaction mechanism of chitin synthase

14.2.3 Regulation

Chitin synthesis is an essential component for insect growth and development. Interruptions in the CHS functions result in developmental disorders. This type of disorders has been studied by Ostrowski et al. (2002) in *Drosophila*. They studied the morphology of mutant *Drosophila* flies with defect in the *krotzkopf verkehrt* gene and proved the importance of CHS in growth and development of insect. Ecdysterone is a common steroid hormone involved in the insect molting and metamorphosis regulation (Merzendorfer 2006). This hormone acts on gene transcription by activating a nuclear heterodimeric receptor. Chitin synthesis, being a cyclic event in insects, aids ecdysterone in controlling the CHS gene expression. There are many studies in the support of the role of 20-hydroxyeedysone and juvenile hormone (Zhu et al. 2016). A study conducted by Arakane et al. (2005)) in the red flour beetle, *Tribolium castaneum*, showed the unique complementary roles of the two CHS genes TcCHS1 and TcCHS2. They proved the vitality of TcCHS1 in all three types of molts and reduction of whole-body chitin content, whereas the TcCHS2 had a role in the downregulation that leads to the shrinkage in larval size and reduced chitin content in the midgut. Ibrahim et al. (2000) studied CHS in mosquitoes and found the expression of various CHS genes at different developmental stage. CHS1 genes are expressed in almost all developmental stages, while CHS2 are expressed only in larval stages. Zhuo et al. (2014) cloned CHS A gene (BmChsA) (Accession Number: JQ320074) from *Bombyx mori*. They observed CHS A gene as an epidermis-specific expressed gene during the molting processes. The gene expression was upregulated by exogenous ecdysone and juvenile hormone. The knockdown of the BmChsA gene shows significant increase in the percentage of non-exuvial and abnormal exuvial larvae. Reduced expression of the BmChsA gene was detected when exposed to the CHS inhibitor nikkomycin Z that leads to the low chitin content in the newly formed epidermal tissue. Tetreau et al. (2015) studied two CHS genes, i.e., MsCHS1 (accession number, AY062175) and MsCHS2 (AY821560) of *Manduca sexta* and reported the expression of MsCHS1 in the head and abdomen of the *M. sexta* larvae and adults (tissues that include epidermal cells involved in cuticle production), while MsCHS2 was exclusively expressed in the midgut. They also predicted that MsCHS2 secreted by the midgut epithelial cells are responsible for the synthesis of chitin in the PM, while MsCHS1 may synthesize chitin present in the epidermal cuticle and the tracheal cuticle (Hogenkamp et al. 2005).

14.2.4 Applications

CHS is the enzyme responsible for the synthesis of chitin in insect exoskeleton. CHS inhibitors are gaining huge importance in therapeutic medicine. CHS has also role in plant infection process. The utilization of pesticides is crucial for crop, public hygiene, and pest control. The present commercial pesticides are facing the problems of resistance. To properly check and control the problems arising from the

insufficient pest control, there is a need to search for novel compounds capable of acting at new target sites. CHS is essential for insect growth and development, and thus it also serves as the ideal target for insecticides.

Arakane et al. (2008) have reported the role of CHS in red flour beetle, *T. castaneum*, and conferred that CHS is required for embryonic and adult development as well as for other types of molting. RNA interference (RNAi)-mediated knockdown of CHS genes can be an efficient pest control approach (Harris and Fuhrman 2002; Mansur et al. 2014; Veronico et al. 2001). Apart from CHS, enzymes like acetylcholinesterase, cytochrome P450, amino peptidase N, allatostatin, allatotropin, tryptophan oxygenase, arginine kinase, etc. can be regulated by RNAi approach (Kola et al. 2015). Zhang et al. (2010), on the basis of the observation that chitin synthesis can be blocked by dsRNA in mosquitoes, established a process to create a systemic knockdown of CHS gene expression in *Anopheles gambiae* larvae by nourishing with nanoparticles containing chitosan and dsRNA specific for the target gene. Yang et al. (2016) showed the potentiality of developing effective insecticides by repressing CHS1 and chitinase10 genes by miR-71 and miR-263 in locusts, which resulted into blocking of molting and alterations in the chitin content. Zhang and Yan Zhu (2013) reported slight in vitro inhibition of *A. gambiae* CHS activity by the employment of diflubenzuron and nikkomycin Z at the concentration of 2.5 μmol/L. But they did not find any in vivo inhibition at any concentration by polyoxin D. In a study conducted by Chen et al. (2013), the full-length cDNA-encoding chitin synthase 2 (BdCHS2) was cloned and characterized *Bactrocera dorsalis*. The study proposed that BdCHS2 could play a major role in the regulation of midgut chitin content and, thus, affects the growth and development of *B. dorsalis*. Peptidyl nucleosides and acylureas are known CHS inhibitors (Abo-Elghar et al. 2004; Wilson and Cryan 1997; Ruiz-Herrera and San-Blas 2003; Tellam and Eisemann 2000). The former contain polyoxins and nikkomycins, whereas the latter exhibit high CHS activity with compounds like diflubenzuron and novaluron (Merzendorfer 2006; De Cock and Degheele 1998; Soltani et al. 1993). Fontoura et al. (2012) have reported the efficiency of chitin synthesis inhibitor compound, novaluron, against organophosphate-resistant *Aedes aegypti*.

14.3 Chitinases

Chitin is widely allocated in insect species especially in ectodermal epithelial tissues, viz., cuticles, trachea, foregut, and hindgut. It is also an important constituent of intestinal peritrophic matrices (PMs) (Moussian 2010). Periodical molting (i.e., degradation of old cuticle and replaced by newly synthesized one) is an essential process for development of insects that allows continued growth and increased body size (Kramer et al. 1993). A cocktail of hydrolytic enzymes, viz., chitinase, β-*N*-acetylglucosaminidases, proteinase, and lipase, is required for digestion of old cuticle prior to ecdysis. Among these enzymes, chitinases play a critical role in insect growth and development (Kramer and Koga 1986; Zhu et al. 2008b; Arakane and Muthukrishnan 2010). Chitinases in insects have evolved for efficient degradation

of the insoluble polysaccharide to soluble oligosaccharides during the molting process (Arakane et al. 2003). *Anopheles gambiae* contains chitinases in integument and midgut, where it is involved in hydrolyzing chitin from the PM (Shen and Jacobs-Lorena 1997). In addition to chitinase, ecdysteroids also are believed to be key compounds in insect molting via inducing chitinase. However, inapt expression of chitinases in insects during development could disrupt cuticle and/or gut physiology. Zhu et al. (2008a) observed that downregulation of group I chitinase from *T. castaneum* (TcCht5) during the larval, pharate pupal, and pupal stages resulted into failure of cuticle (old) shedding and death during eclosion.

Insect chitinases belong to family 18 of glycoside hydrolase (GH18) and hydrolyze chitin by an endo-mechanism that retains the anomeric β-(1,4) configuration of products (Kramer and Muthukrishnan 2005). Chitinases and chitinase-like proteins in insects have been classified into eight groups on the basis of their catalytic domains, among which only group I is well characterized and mainly involved in chitinolytic processes that have close association with molting (Zhu et al. 2008b; Arakane and Muthukrishnan 2010). Chitinases show enormous variability in their domain structure, enzymatic properties, expression patterns, and tissue localizations. These highly diverse enzymes are encoded by various genes as evident by insect transcriptomes or genomes (Zhu et al. 2004, 2008a; Nakabachi et al. 2010; Pan et al. 2012). *Drosophila melanogaster*, *T. castaneum*, and *A. gambiae* possess 16, 22, and 20 chitinase and chitinase-like genes, respectively (Zhang et al. 2011). These genes show significant variation in size, developmental, and tissue expression patterns. The proteins deduced by these genes also differ in their primary structures and domain architectures (Arakane and Muthukrishnan 2010; Zhang et al. 2011). Interest in chitinases has increased in view of their possible uses as selective biopesticides (Kramer and Muthukrishnan 1997).

14.3.1 Catalytic Mechanism

Substrate-assisted catalysis model was previously opted to unfold the catalytic mechanism of insect chitinases (Fukamizo 2000). But degradation of substrates lacking the *N*-acetyl groups can't be explained by aforesaid mechanism (Honda et al. 2003). Hence, further studies are needed based on the catalytic and chitin-binding domains along with the identification of critically required conserved amino acid residues. Degradation of insoluble insect chitin by chitinase is a multistep dynamic process that essentially follows adsorption via a substrate-binding domain, hydrolysis, desorption, and repositioning of the catalytic domain on the surface of the substrate. Both binding and degradation mechanisms are important to be researched to fully elucidate the catalytic mechanism. Complete metabolism of chitin to *N*-acetylglucosamine requires a concoction of chitinase and *N*-acetylglucosaminidase (Filho et al. 2002). Substrate-assisted catalysis involving a double-displacement mechanism with retention of the anomeric configuration at the cleavage site has been observed as the most favorable mechanism by chitinases (Zechel and Withers 2000; Brameld et al. 2002). Further, inhibition of family

18 chitinases by a transition state analog inhibitor, allosamidin, confirmed the employment of retention mechanism (Brameld et al. 2002; Bortone et al. 2002; Lu et al. 2002). Allosamidin is used as diagnostic compound for enzymes that exhibit retention mechanism. Chitinases from *B. mori* and *Serratia marcescens* were detected to catalyze chitin degradation via retention mechanism (Abdel-Banat et al. 1999; Aronson et al. 2003). *S. marcescens* chitinase follows a processive mechanism for degradation of polysaccharides.

Substrate binding and catalysis by family 18 chitinases require the presence of acidic and aromatic amino acid residues in the catalytic site. Site-directed mutagenesis and functional analysis revealed the presence of three highly conserved acidic amino acid residues D142, D144, and E146 in chitinase from *M. sexta* (Lu et al. 2002). These amino acids were believed to play key roles in catalysis, but none of these were observed to be essential for substrate binding. E146 probably functions as an acid/base catalyst during hydrolysis, while D144 apparently works as an electrostatic stabilizer of the positively charged transition state. The functions of E146 and D144 are influenced by D142 as it affects the *pKa* values of these acidic amino acids.

14.3.2 Molecular Architecture of Chitinase Gene

Insect chitinases possess multi-domain architecture which includes catalytic domains, cysteine-rich chitin-binding domains, fibronectin-like domains, mucin-like domains, and Ser-/Thr-rich linker domains (glycosylated) along with a signal peptide (Tellam 1996; Suzuki et al. 1999). Chitinase 5 from *Tenebrio molitor* has a proline-/threonine-rich domain, putative chitin-binding domains, and catalytic domains. Chitin-binding domains are potentially involved in the anchoring of the enzyme to the cuticle (Royer et al. 2002). *M. sexta* (tobacco hornworm) chitinase is a glycoprotein, consists of an *N*-terminal catalytic domain, a Ser-/Thr-rich linker region, and a *C*-terminal chitin-binding domain (Arakane et al. 2003). The activities of catalytic and chitin-binding domains were independent of each other and function separately. Attachment of chitin-binding domain to the catalytic domain enhanced the activity of the latter toward the insoluble polymer but not for the soluble chitin oligosaccharides. The enhancement in catalytic activity primarily attributed to the increased affinity (decreased K_m) of enzyme for the insoluble substrate. Linker region plays dual roles by stabilizing the enzyme in the presence of gut proteolytic enzymes and facilitating its secretion from the cell. Both the linker region and catalytic domain exhibit *O*-linked and *N*-linked glycosylation, but the degree of modification varies from moderate to extensive. The linker region along with elements of the chitin-binding domain works as a major immunogenic epitope. *M. sexta* chitinase contains an active site amino acid residue W145 as evident by protein modeling and substrate-docking experiments (Huang et al. 2000). W145 is essential for enzyme to attain optimal catalytic activity but not for chitin binding. Kinetic studies revealed that W145 not only increases the affinity of the enzyme for the polymeric substrate but also extends the enzyme's activity at alkaline pH range.

Locusta migratoria (migratory locust) shows duplication of chitinase 5 (Cht5) into two to five different genes (LmCht5-1 and LmCht5-2) (Li et al. 2015a). Structural and phylogenetic analyses suggested orthology of LmCht5-1 to other insect Cht5 genes, whereas LmCht5-2 might be newly duplicated. Both duplicated genes LmCht5-1 and LmCht5-2 possess a signal peptide and a catalytic domain with four conserved motifs and contain 505 and 492 amino acids, respectively. However, chitin-binding domain is present only in LmCht5-1. Chitinases are considered as the critical cuticle-degrading enzymes in crustaceans. Chinese mitten crab, *Eriocheir sinensis*, genome encoded five chitinase genes, viz., EsCht1, EsCht2, EsCht3, EsCht4, and EsCht6 (Li et al. 2015b). The open reading frames (ORF) of EsCht1, EsCht2, EsCht3, EsCht4, and EsCht6 ranged from 1182 to 1926 bp encoding 393 and 641 amino acid residue long proteins. Domain analysis of *E. sinensis* chitinase genes revealed that most EsChts contained the catalytic domain and the chitin-binding domain (CBD) connected with the serine/threonine (S/T)-rich linker region. Phylogenetic analyses revealed that EsChts with orthologs in crustaceans were divided into six groups. Chitinase genes showed tissue-dependent, developmental stage-related, and molting stage-related differential expression patterns.

Chitinolytic enzymes have physiological significance in the immune and digestive systems in plants and animals. The Ca-Chit protein from *Crassostrea angulata* (119 kDa) contains a glycoside hydrolase family 18 domain, threonine-rich region profile, and a putative membrane anchor domain with considerable homology with other family 18 chitinases (Yang et al. 2015). Ca-Chit first expresses in the visceral mass of larvae and then in the digestive gland forms a crystalline structure during larval development.

14.3.3 Cloning of Chitinase Gene

Chitinase genes from various insect species have been cloned and studied to get more information about structure, gene variants, and related functions. Kim et al. (1998) cloned the cDNAs encoding chitinases from *B. mori* and *Hyphantria cunea* and studied their gene expression during metamorphosis. Southern blot analysis further divulged that *B. mori* genome contains only one chitinase gene, while *H. cunea* genome has one or two chitinase gene copies. The chitinase cDNA of *B. mori* and *H. cunea* encoded 63.4 and 62 kDa proteins that contain 565 and 553 amino acids, respectively. Both the chitinases exhibit 75% and 77–80% homology with chitinase from *M. sexta*. The enzymes were *N*-glycosylated, and the *B. mori* enzyme has three potential *N*-glycosylation sites at the amino acid residues 86–89, NFTS; 304–307, NATG; and 398–401, NYTV, whereas two potential sites were present at the amino acid residues 86–89, NFTA and 304–307, NATG in *H. cunea* chitinase. The presence of amino acid residues at conserved positions is necessarily required for activation of chitinases that secreted as inactive zymogens in insects. Lysine or arginine residues are present at conserved positions in the *Tenebrio molitor* chitinase 5 (Royer et al. 2002). Similarly, lysine residues are also important for activation of *A. gambiae* chitinases (Shen and Jacobs-Lorena 1997).

cDNA-encoding chitinase from larvae of tomato moth (*Lacanobia oleracea*), a lepidopteran pest of crops, was cloned by Fitches et al. (2004). The enzyme exhibited 75–80% identity with other lepidopteran chitinases, which contain 553 amino acid residues with a signal peptide of 20 amino acids and required for molting. Administration of chitinase prior to molting resulted into decreased cuticle thickness, larval growth, and food consumption (>50%). Chitinase (OfCht5) from *Ostrinia furnacalis* was cloned and expressed in yeast *Pichia pastoris* (Wu et al. 2013). cDNA sequence analysis suggested that OfCht5 belongs to group I chitinases. Yang et al. (2015) cloned and characterized novel chitinase homologue, Ca-Chit from the oyster *C. angulata*. The full-length (3998bp) cDNA of Ca-Chit consisted of 23bp 5-UTR, 3288 ORF, and 688bp 3-UTR. The study proved the importance of chitinase in molluscan digestive system. Chitinase gene Ifu-chit2 from *Isaria fumosorosea* was cloned and characterized by Meng et al. (2015). 1435-bp-long Ifu-chit2 gene was encoded for a 423 amino acid polypeptide and interrupted by three short introns. The Ifu-Chit2 protein showed homology with *Beauveria bassiana* chitinase (Bb-chit2). In vivo expression of Ifu-chit2 suggested that chitinase may play a role in the early stage of pathogenesis.

14.3.4 Expression of Chitinase Gene and Development

In insects, chitinase-encoding genes are differentially expressed during development, with some genes having restricted expression in certain tissues. Chitinase expression profiles of *T. castaneum*, *A. gambiae*, and *Acyrthosiphon pisum* have been studied in detail employing reverse transcription and qualitative polymerase chain reaction (Zhu et al. 2008b; Nakabachi et al. 2010; Zhang et al. 2011). The presence of Cht5 transcripts in the epidermis and gut of *Helicoverpa armigera* suggested their possible involvement in chitin turnover-associated tissues, viz., cuticular exoskeleton and peritrophic membrane (Ahmad et al. 2003). Importance of Cht5 in development was confirmed in *T. castaneum* and *Spodoptera exigua* by RNAi technology (Zhu et al. 2008b; Zhang et al. 2012).

Upregulation of chitinase gene expression in *B. mori* and *Hyphantria cunea* was detected during molting, larval–pupal transformation, and pupal–adult transformation (Kim et al. 1998). Expression of *T. molitor* chitinase 5 (TmChit5) is associated with the metamorphosis and regulated by two hormones, viz., 20hydroxyecdysone (20E) and juvenile hormone (JH) (Royer et al. 2002). TmChit5 speculated to be secreted as a zymogen, which may be cleaved by trypsin-like enzymes, presents in the molting fluid and generates active units 3, 4, and 5. The presence of PEST sequences can further enhance the proteolysis. The enzyme incorporated into the old cuticle through its four chitin-binding domains (Bade 1974).

Chitinases were observed to be present as inactive zymogens in various insect species which subsequently activated by proteolysis. In the integument of *B. mori*, an enzyme with putative chitinase activity had been observed to be secreted as a zymogen (Koga et al. 1992). In *M. sexta*, the presence of chitinases is detected in the molting fluid and is associated with the degradation of the old cuticle. Appearance

of proteolytic enzymes in the molting fluid that coincided with the beginning of degradation suggested the possible role of proteolysis in activation of chitinases (Samuels and Reynolds 1993). Similar results were detected in (Shen and Jacobs-Lorena 1997) *A. gambiae*, where whole gut extracts show chitinase activity after treatment with trypsin that cleaves at Lys-31 or Lys-32. Lysine or arginine residues are also present at conserved positions in the *T. molitor* chitinase 5.

Multiple chitinases are observed to be produced by entomopathogenic fungi, and some of them act synergistically with proteases to degrade insect cuticle. However, involvement of chitinase in insect fungus pathogenesis has not been fully decoded. A 33 kDa endochitinase, Bbchit1, was produced by *Beauveria bassiana*; Bbchit1 exhibited significant similarity to *Trichoderma harzianum* endochitinase Chit36Y and putative endochitinase from *Streptomyces avermitilis* and *Streptomyces coelicolor* (Fang et al. 2005). However, Bbchit1 had low identity levels to other chitinase genes from entomopathogenic fungi. The presence of putative CreA/Crel carbon catabolic repressor-binding domains in the regulatory sequence was consistent with glucose suppression of Bbchit1. Insect bioassays indicated that the enhanced expression of Bbchit1 is related with the increased virulence of *B. bassiana* for aphids.

Chitinase family genes in insects are functionally specialized, primarily during the molting process. Among the large family of chitinase-like proteins present in the red flour beetle, *T. castaneum* (Tc), TcCHT5 was found to be required only for pupal–adult molting (Zhu et al. 2008a). Downregulation of TcCHT5 by injection of enzyme-specific dsRNA into larvae resulted into a lethal phenotype. The larvae had metamorphosed into pupae and then pharate adults with incomplete adult eclosion. Deterrence of embryo hatch, larval molting, pupation, and adult metamorphosis by specific knockdown of TcCHT10 indicated its vital role these processes. Another chitinase-like protein, TcCHT7, was essentially required for abdominal contraction and wing/elytra extension immediately after pupation, while its role was dispensable for larval–larval molting, pupation, and adult eclosion. TcIDGF4 is also a chitinase-like protein that contributed to adult eclosion. Knockdown of other chitinase-like proteins like imaginal disk growth factor 2 (IDGF2) has not resulted into phenotypic effects. The studies on *T. castaneum* chitinases provided a biological rationale for the presence of a large assortment of chitinase-like proteins.

Locusta migratoria secreted duplicated chitinase 5 genes among which LmCht5-1 was observed to be expressed in hindgut while LmCht5-2 in integument, foregut, hindgut, and fat bodies (Li et al. 2015a). Furthermore, LmCht5-2 is not vital for development and survivorship of the locust. Similar expression patterns of LmCht5-1 and LmCht5-2 from the fourth-instar nymphs to the adults suggested their similar regulation and response to the active molting hormone, 20-hydroxyecdysone.

Chitinase variants from *E. sinensis* exhibit differential expression patterns implied to their distinct biological functions during growth, development, and reproductive stages (Li et al. 2015b). EsCht1 from group I might play a role in the digestion of chitin-containing food, while EsCht2 from group III has a role in the degradation of chitinous cuticle during molting for growth and during the

postembryonic development. EsCht3 from group III potentially had dual roles in the digestion of chitin-containing food and defense against chitin-bearing pathogens. EsCht3, EsCht4, and EsCht6 are potentially required reproductive molting as evident by their overexpression in the reproductive system. Enhanced EsCht2 mRNA expression in the cuticle and EsCht4 and EsCht6 mRNA expression in the hepatopancreas were 108-fold, 19-fold, and 12-fold which was observed in the premolt as compared to the intermolt stage, respectively, during molting (Li et al. 2015b).

Sex-related variations in properties of chitin present in male and female grasshopper species revealed that the α-chitin present in all is similar in terms of thermal properties and crystalline index (Kaya et al. 2015). Two major differences observed in chitin with respect to gender were the presence of more amount of chitin in males than females with dry weight ranging from 4.71% to 11.84% and occurrence of nanofibers (25–90 nm) and nanopores (90–250 nm) in the male chitin surface. Nanofibers were observed only in *Melanogryllus desertus* females.

The cuticle forms an apical extracellular matrix (ECM) that covers exposed organs, such as the epidermis, trachea, and gut, for organizing morphogenesis and protection of insects. Cuticle proteins and chitin are involved in the formation of extracellular matrix. Chitinases (Chts) and imaginal disc growth factors (Idgfs) were observed to be essential for larval and adult molting in *Drosophila* (Pesch et al. 2016). Depletion of Cht and Idgf resulted into deformed cuticle, larval, and adult molting defects and insufficient protection against wounding and bacterial infection that led to early lethality. *Cht2*/*Cht5*/*Cht7*/*Cht9*/*Cht12* and idgf1/idgf3/idgf4/idgf5/idgf6 are needed for organizing proteins and chitin matrix at the apical cell surface (Pesch et al. 2016). Chts are required for extracellular matrix formation, while idgfs act as structural proteins to maintain the extracellular matrix scaffold against chitinolytic degradation. Chts and Idgfs play analogous roles in ECM dynamics across the insect taxa put forwarded them as new targets for species-specific pest control.

Chitin has also been identified in the compound eyes of arthropods, where it is considered as a part of the visual system. Corneal lens of dragonfly (*Sympetrum fonscolombii*) contains 20.3% chitin and presents the α-form for increased mechanical strength (Kaya et al. 2016a). Scanning electron micrographs revealed that the outer part of corneal lenses consisted of long chitin fibrils with regular arrays of papillary structures, while the smoother inner part had concentric lamellated chitin formation with shorter chitin nanofibrils. The presence of chitin in the compound eyes paves the way to design chitin-based optical materials. Physicochemical characteristics and chitin content were observed to variate with developmental stages in *Vespa crabro* (Kaya et al. 2016b). With the growth, chitin content of *V. crabro* was gradually increased from 2.1% to 10.3% with altered surface properties. From larval stage to pupal stage, threefold increase in chitin deposition was observed. The house dust mite (HDM) allergen Der p 18 belongs to the glycoside hydrolase family 18 chitinases (Resch et al. 2016). The allergen exhibits weak chitin-binding activity and is mainly present in the PM of HDM gut and to a lower extent in fecal pellets. The allergen can be utilized for developing diagnostic test panels for HDM allergy.

14.3.5 Crystal Structure of Insect Chitinases

Scant information is available on crystal structure of insect chitinases. Chen et al. (2014a) studied crystal structures of unliganded and oligosaccharide-complexed chitinase from *Ostrinia furnacalis* (OfChtI). The enzyme is believed to be essential for molting. OfChtI contains a long substrate-binding cleft similar to the bacterial exoacting chitinase from *S. marcescens* (SmChiB). However, unlike OfChtI which possesses an open and groove-like cleft, SmChiB has a blocked and tunnel-like cleft. OfChtI is an endo-acting enzyme and acts from nonreducing ends. The presence of reducing sugar at subsite 1 is an energetically unfavored "boat" conformation, as evident by complexed structure of the catalytic domain of OfChtI (OfChtI-CAD) with (GlcNAc) 2/3. A hydrophobic plane composed of four surface-exposed aromatic residues is present near to the substrate-binding cleft, which are essential for chitin-binding activity. The role of a series of fully deacetylated chitooligosaccharides (GlcN)2e7 as inhibitors of OfCht5 has been demonstrated by Chen et al. (2014b).

14.3.6 Chitinase-Like Proteins

A range of chitinase-like proteins are also present in various insect species that lack catalytic activity. Insect chitinase-like proteins were observed to be encoded by a large and diverse group of genes as suggested by bioinformatics-based investigation of three insect species, i.e., *D. melanogaster*, *A. gambiae*, and *T. castaneum* (Zhu et al. 2008c). Sixteen, 16, and 13 putative chitinase-like genes have been identified in the genomic databases of *T. castaneum*, *D. melanogaster*, and *A. gambiae*, respectively. Based on the phylogenetic analyses, the chitinase-like proteins have been classified into five groups. Among the groups, groups I–III are each represented by only a single gene in each species, while multiple genes encode group IV and group V chitinase-like proteins. Group I chitinases are secretory proteins, are abundant in molting fluid and/or integument, and contain single copy of catalytic and chitin-binding domain (ChBD) connected by an S-/T-rich linker polypeptide. Group II chitinases possess multiple catalytic and ChBDs and are unusually larger-sized secreted proteins. Group III chitinases are predicted to be membrane-anchored proteins that contain two catalytic domains. The most divergent is group IV chitinases that usually lack a ChBD and/or an S-/T-rich linker domain. These are present as secreted proteins in gut or fat body. Putative chitinase-like imaginal disc growth factors (IDGFs) included in the group V.

Glycoside hydrolase family 18 involves chitinase-like proteins (CLPs) that possess structural similarity to chitinases but lack enzymatic activity (Kucerova et al. 2016). CLPs are observed to be upregulated in several human disorders that affect regenerative and inflammatory processes, but very little is known about their normal physiological function. Kucerova et al. (2016) showed imaginal disc growth factor 3 (IDGF3), a CLP from *D. melanogaster*, which plays an immune-protective role during entomopathogenic nematode infections. Whole-genome transcriptional

analysis of nematode-infected wild type and Idgf3 mutant larvae revealed that IDGF3 also has roles in repression of Jak/STAT and wingless signaling. IDGF3 has multiple roles in innate immunity. It is an essential component required for the formation of hemolymph clots to seal wounds. Vertebrate and invertebrate CLP proteins supposed to have analogous function and a broad impact on inflammatory reactions and infections. Further, genetic analysis of CLP will help to elucidate molecular basis of CLP functions.

14.3.7 Chitinases as Biocontrol Agent

The importance of chitinolytic enzymes for insect, nematode, and fungal growth has raised the concerns regarding their employment not only as biopesticides or chemical defense proteins in transgenic plants but also as microbial biocontrol agents. Insect pests and pathogenic fungi can be biologically controlled by targeting chitin present in their extracellular matrices and cell wall. Chitinases can be exploited as insect control agents due to their ability to degrade chitin associated with the PM or exoskeleton. cDNA of chitinase from cotton leaf worm (*Spodoptera littoralis*) has been synthesized, and its tolerance against insects was increased by transgenic maize plant system (Osman et al. 2015). Insect chitinase transcripts and proteins were expressed in transgenic maize plants, and their functional integrity was confirmed using insect bioassays. The bioassays employing transgenic maize plants against *Sesamia cretica* (corn borer) revealed that ~50% of the insects present on transgenic plants were died owing to their enhanced resistance against *S. cretica*.

In planta expression of chitinase RNAi effectors using a recombinant plant virus can be potentially employed to control *Mythimna separata*, a prevalent corn pest in China (Bao et al. 2016). Fragments of the *M. separata* chitinase sequences were cloned into a virus vector in order to produce RNA interference (RNAi) effectors during virus infection and replication in plants. Expression of target MseChi1 and MseChi2 genes were downregulated by 76 and 45 %, respectively, when the infected plants were fed to *M. separata*.

The filamentous fungus, *I. fumosorosea*, is a chitinase producing promising insect biocontrol agent (Huang et al. 2016). The chitinase (chit1) gene from *I. fumosorosea* encoded for a 423 amino acid (46kDa) long protein present as a single copy in the genome and an important virulence factor. Chit1 gene knockout strains of *I. fumosorosea* (ΔIfchit1) displayed minor alterations in mycelial growth, increased temperature sensitivity, delayed sporulation, and increased conidial production. Also, ΔIfchit1 exhibits decreased infectivity, i.e., increased LC50 (threefold to fourfold) and a significantly delayed time to death (LT50 from 3 to 6 days) as revealed by insect bioassays using *Plutella xylostella* (diamond back moth). Chitinase (HaCHI) gene was selected as a potential target to develop sustainable and environmental-friendly methods for crop improvement and protection against devastating agricultural insect pest, *H. armigera* (Reddy and Rajam 2016). The enzyme is critically required for molting and metamorphosis.

14.4 Conclusions and Future Perspectives

Chitozymes have been thoroughly explored during the past decades because of their wide spectrum of applications and growing interests in insect biotechnology. Chitin is an important part of insect exoskeleton and PM, whereas chitinase appeared as a critical enzyme for growth and development. Apart from chitin, many other components are also present in insect cuticle. Interaction of chitin with other components of extracellular matrix will redefine the integrity of chitin as an essential exoskeleton component. Expression of chitin synthesizing and metabolizing enzymes is associated with various stages of insect growth and development advocating their diverse physiological functions. Research on the multiplicity of chitin synthases and chitinases will provide an idea about their relation with insect growth. Cloning of the genes would provide recombinant enzymes and sub-domains, which can be utilized to study regulation and specific functions of multiple enzyme forms. Chitin synthases are comparatively less studied enzymes, and because of that the research on chitin biosynthesis is still in its infancy. Initiation and elongation processes whether involved along with the essential precursor molecules need to be explored to understand the detailed mechanism of chitin synthesis. This would also support development of novel insecticidal agents. Catalytic mechanism of multiple enzyme forms will give further insight to the chitin synthesis and degradation in relation to the molting. Furthermore, exploration and characterization of chitinase-like proteins may provide some novel targets to be exploited as biocides. The significance of chitinases as biocontrol agents can further be explored by developing transgenic plants expressing chitinase genes against insect and plant pathogens. Enhancing the gene expression levels along with combination of other insecticidal agents may further improve the potentiality of chitinase in biocontrol of pests and pathogens. Moreover, improved understanding of structure, catalysis, and biochemistry of these largely unexploited resources will accelerate their utilization in various biotechnological processes.

References

Abdel-Banat BMA, Kameyama Y, Yoshioka T, Koga D (1999) Purification and characterization of a 54 kDa chitinase from *Bombyx mori*. Insect Biochem Mol Biol 30:107–117

Abo-Elghar GE, Fujiyoshi P, Matsumura F (2004) Significance of the sulfonylurea receptor (SUR) as the target of diflubenzuron in chitin synthesis inhibition in *Drosophila melanogaster* and *Blattella germanica*. Insect Biochem Mol Biol 34:743–752

Ahmad T, Rajagopal R, Bhatnagar RK (2003) Molecular characterization of chitinase from polyphagous pest *Helicoverpa armigera*. Biochem Biophys Res Commun 310:188–195

Arakane Y, Muthukrishnan S (2010) Insect chitinase and chitinase-like proteins. Cell Mol Life Sci 67:201–216

Arakane Y, Zhu Q, Matsumiya M, Muthukrishnan S, Kramer KJ (2003) Properties of catalytic, linker and chitin-binding domains of insect chitinase. Insect Biochem Mol Biol 33:631–648

Arakane Y, Hogenkamp DG, Zhu YC et al (2004) Characterization of two chitin synthase genes of the red flour beetle, *Tribolium castaneum*, and alternate exon usage in one of the genes during development. Insect Biochem Mol Biol 34:291–304

Arakane Y, Muthukrishnan S, Kramer K et al (2005) The Tribolium chitin synthase genes TcCHS1 and TcCHS2 are specialized for synthesis of epidermal cuticle and midgut peritrophic matrix. Insect Mol Biol 14:453–463

Arakane Y, Specht CA, Kramer KJ et al (2008) Chitin synthases are required for survival, fecundity and egg hatch in the red flour beetle, *Tribolium castaneum*. Insect Biochem Mol Biol 38:959–962

Aronson NN, Halloran BA, Alexyev ME, Amable L, Madura JD (2003) Family 18 chitinase oligosaccharide substrate interaction: subsite preference and anomer selectivity of *S. marcescens* chitinase A. J Biochem 376:87–95

Bade ML (1974) Localisation of molting chitinase in insect cuticle. Biochim Biophys Acta 372:474–477

Bao W, Cao B, Zhang Y, Wuriyanghan H (2016) Silencing of *Mythimna separata* chitinase genes via oral delivery of in planta-expressed RNAi effectors from a recombinant plant virus. Biotechnol Lett 38:1961–1966. doi:10.1007/s10529-016-2186-0

Bortone K, Monzingo AE, Ernst S, Robertus JD (2002) The structure of an allosamidin complex with the *Coccidioides immitis* chitinase defines a role for a second acid residue in substrate-assisted mechanism. J Mol Biol 320:293–302

Brameld KA, Shrader WD, Imperiali B, Goddard WA (2002) Substrate assistance in the mechanism of family 18 chitinases: theoretical studies of potential intermediates and inhibitors. J Mol Biol 280:913–923

Chapman RF (1998) The insects: structure and function. Cambridge University Press, New York

Chen AC (1987) Chitin metabolism. Arch Insect Biochem Physiol 6:267–277

Chen L, Yang W-J, Cong L et al (2013) Molecular cloning, characterization and mRNA expression of a chitin synthase 2 gene from the oriental fruit fly, *Bactrocera dorsalis* (Diptera: Tephritidae). Int J Mol Sci 14:17055–17072

Chen L, Liu T, Zhou Y, Chen Q, Shen X, Yang Q (2014a) Structural characteristics of an insect group I chitinase, an enzyme indispensable to moulting. Acta Crystallogr D Biol Crystallogr 70:932–942

Chen L, Zhou Y, Qu M, Zhao Y, Yang Q (2014b) Fully deacetylated chitooligosaccharides act as efficient glycoside hydrolase family 18 chitinase inhibitors. J Biol Chem 289:17932–17940

Cohen E (1987) Chitin biochemistry: synthesis and inhibition. Annu Rev Entomol 32:71–93

Coutinho PM, Deleury E, Davies GJ et al (2003) An evolving hierarchical family classification for glycosyltransferases. J Mol Biol 328:307–317

De Cock A, Degheele D (1998) Buprofezin: a novel chitin synthesis inhibitor affecting specifically planthoppers, whiteflies and scale insects. In: Insecticides with novel modes of action. Springer, Berlin Heidelberg, pp 74–91

Demaeght P, Osborne EJ, Odman-Naresh J et al (2014) High resolution genetic mapping uncovers chitin synthase-1 as the target-site of the structurally diverse mite growth inhibitors clofentezine, hexythiazox and etoxazole in *Tetranychus urticae*. Insect Biochem Mol Biol 51:52–61

Dorfmueller HC, Ferenbach AT, Borodkin VS et al (2014) A structural and biochemical model of processive chitin synthesis. J Biol Chem 289:23020–23028

Fang W, Leng B, Xiao Y, Jin K, Ma J, Fan Y, Feng J, Yang X, Zhang Y, Pei Y (2005) Cloning of *Beauveria bassiana* chitinase gene *bbchit1* and its application to improve fungal strain virulence. Appl Environ Microbiol 71:363–370

Filho BPD, Lemos EA, Secundino NEC, Pascoa V, Pereira ST (2002) Presence of chitinase and β -*N*-acetylglucosaminidase in the *Aedes aegypti* chitinolytic system involving peritrophic matrix formation and degradation. Insect Biochem Mol Biol 32:1723–1729

Fitches E, Wilkinson H, Bell H, Bown DP, Gatehouse JA, Edwards JP (2004) Cloning, expression and functional characterisation of chitinase from larvae of tomato moth (*Lacanobia oleracea*): a demonstration of the insecticidal activity of insect chitinase. Insect Biochem Mol Biol 34:1037–1050

Fontoura NG, Bellinato DF, Valle D et al (2012) The efficacy of a chitin synthesis inhibitor against field populations of organophosphate-resistant *Aedes aegypti* in Brazil. Mem Inst Oswaldo Cruz 107:387–395

Fukamizo T (2000) Chitinolytic enzymes: catalysis, substrate binding, and their application. Curr Protein Pept Sci 1:105–124

Harris MT, Fuhrman JA (2002) Structure and expression of chitin synthase in the parasitic nematode *Dirofilaria immitis*. Mol Biochem Parasitol 122:231–234

Hogenkamp DG, Arakane Y, Zimoch L et al (2005) Chitin synthase genes in *Manduca sexta*: characterization of a gut-specific transcript and differential tissue expression of alternately spliced mRNAs during development. Insect Biochem Mol Biol 35:529–540

Honda Y, Kitaoka M, Tokuyasu K, Sasaki C, Fukamizo T et al (2003) Kinetic studies on the hydrolysis of *N*-acetylated and *N*-deacetylated derivatives of 4-methylumbelliferyl chitobioside by the family 18 chitinases ChiA and ChiB from *Serratia marcescens*. J Biochem (Tokyo) 133:253–258

Huang X, Zhang H, Zen KC, Muthukrishnan S, Kramer KJ (2000) Homology modeling of the insect chitinase catalytic domain–oligosaccharide complex and the role of a putative active site tryptophan in catalysis. Insect Biochem Mol Biol 30:107–117

Huang Z, Hao Y, Gao T, Huang Y, Ren S, Keyhani NO (2016) The Ifchit1 chitinase gene acts as a critical virulence factor in the insect pathogenic fungus *Isaria fumosorosea*. Appl Microbiol Biotechnol 100:5491–5503

Ibrahim GH, Smartt CT, Kiley LM et al (2000) Cloning and characterization of a chitin synthase cDNA from the mosquito *Aedes aegypti*. Insect Biochem Mol Biol 30:1213–1222

Kaya M, Tozak KÖ, Baran T et al (2013) Natural porous and nano fiber chitin structure from *Gammarus argaeus* (Gammaridae Crustacea). Excli J (In eCollection) 12:503–510

Kaya M, Lelesius E, Nagrockaite R, Sargin I, Arslan G, Mol A, Baran T, Can E, Bitim B (2015) Differentiations of chitin content and surface morphologies of chitins extracted from male and female Grasshopper species. PLoS One 10:1–14

Kaya M, Sargin I, Al-jafa I, Erdogan S, Arslan G (2016a) Characteristics of corneal lens chitin in dragonfly compound eyes. Int J Biol Macromol 89:54–61

Kaya M, Sofi K, Sargin I, Mujtabaa M (2016b) Changes in physicochemical properties of chitin at developmental stages (larvae, pupa and adult) of *Vespa crabro* (wasp). Carbohydr Polym 145:64–70

Kim MG, Shin SW, Bae KS, Kim SC, Park HY (1998) Molecular cloning of chitinase cDNAs from the silkworm, *Bombyx mori* and the fall webworm, *Hyphantria cunea*. Insect Biochem Mol Biol 28:163–171

Koga D, Funakoshi T, Mizuki K, Ide A, Kramer KJ, Zen KC, Choi H, Muthukrishnan S (1992) Immunoblot analysis of chitinolytic enzymes in integument and molting fluid of the silkworm *Bombyx mori* and the tobacco hornworm *Manduca sexta*. Insect Biochem Mol Biol 22:305–311

Kola VSR, Renuka P, Madhav MS et al (2015) Key enzymes and proteins of crop insects as candidate for RNAi based gene silencing. Front Physiol 6:119–134

Kramer KJ, Koga D (1986) Insect chitin: physical state, synthesis, degradation and metabolic regulation. Insect Biochem 16:851–877

Kramer KJ, Muthukrishnan S (1997) Insect chitinases: molecular biology and potential use as biopesticides. Insect Biochem Mol Biol 27:897–900

Kramer KJ, Muthukrishnan S (2005) Chitin metabolism in insects. In: Gilbert LI, Iatrou K, Gill S (eds) Comprehensive molecular insect science, vol 4. Elsevier, Oxford, pp 111–144

Kramer K, Muthukrishnan S (2009) Chitin metabolism in insects. In: Gillbert LI (ed) Insect development: morphogenesis, molting and metamorphosis. Academic Press, London, pp 497–530

Kramer KJ, Corpuz LM, Choi H, Muthukrishnan S (1993) Sequence of a eDNA and expression of the gene encoding epidermal and gut chitinases of *Manduca sexta*. Insect Biochem Mol Biol 23:691–701

Kucerova L, Broz V, Arefin B, Maaroufi HO, Hurychova J, Strnad H, Zurovec M, Theopold U (2016) The *Drosophila* chitinase-like protein IDGF3 is involved in protection against nematodes and in wound healing. J Innate Immun 8:199–210

Li D, Zhang J, Wang Y, Liu X, Ma E, Sun Y, Li S, Zhu KY, Zhang J (2015a) Two chitinase 5 genes from *Locusta migratoria*: molecular characteristics and functional differentiation. Insect Biochem Mol Biol 58:46–54

Li X, Xu Z, Zhou G, Lin H, Zhou J, Zeng Q, Mao Z, Gu X (2015b) Molecular characterization and expression analysis of five chitinases associated with molting in the Chinese mitten crab, *Eriocheir sinensis*. Comp Biochem Physiol B 187:110–120

Lombard V, Ramulu HG, Drula E et al (2014) The carbohydrate-active enzymes database (CAZy) in 2013. Nucleic Acids Res 42:D490–D495

Lu Y, Zen KC, Muthukrishnan S, Kramer KJ (2002) Site-directed mutagenesis and functional analysis of active site acidic amino acid residues D142, D144 and E146 in *Manduca sexta* (tobacco hornworm) chitinase. Insect Biochem Mol Biol 32:1369–1382

Mansur JF, Alvarenga ES, Figueira-Mansur J et al (2014) Effects of chitin synthase double-stranded RNA on molting and oogenesis in the Chagas disease vector *Rhodnius prolixus*. Insect Biochem Mol Biol 51:110–121

Meng H, Wang Z, Meng X, Xie L, Hunag B (2015) Cloning and expression analysis of the chitinase gene Ifu-chit2 from *Isaria fumosorosea*. Genet Mol Biol 38:381–389

Merzendorfer H (2006) Insect chitin synthases: a review. J Comp Physiol 176:1–15

Merzendorfer H (2011) The cellular basis of chitin synthesis in fungi and insects: common principles and differences. Eur J Cell Biol 90:759–769

Merzendorfer H, Zimoch L (2003) Chitin metabolism in insects: structure, function and regulation of chitin synthases and chitinases. J Exp Biol 206:4393–4412

Morgan JL, Strumillo J, Zimmer J (2013) Crystallographic snapshot of cellulose synthesis and membrane translocation. Nature 493:181–186

Moussian B (2008) The role of GlcNAc in formation and function of extracellular matrices. Comp Biochem Physiol B Biochem Mol Biol 149:215–226

Moussian B (2010) Recent advances in understanding mechanisms of insect cuticle differentiation. Insect Biochem Mol Biol 40:363–375

Moussian B, Schwarz H, Bartoszewski S et al (2005) Involvement of chitin in exoskeleton morphogenesis in *Drosophila melanogaster*. J Morphol 264:117–130

Muthukrishnan S, Merzendorfer H, Arakane Y, Kramer KJ (2012) Chitin metabolism in insects. In: Insect molecular biology and biochemistry. Elsevier, London, pp 193–235. doi:10.1016/B978-0-12-384747-8.10007-8

Muthukrishnan S, Merzendorfer H, Arakane Y et al (2016) Chitin metabolic pathways in insects and their regulation. In: Extracellular composite matrices in arthropods. Springer, Cham, pp 31–65

Nakabachi A, Shigenobu S, Miyagishima S (2010) Chitinase-like proteins encoded in the genome of the pea aphid, *Acyrthosiphon pisum*. Insect Mol Biol 19:175–185

Osman GH, Assem SK, Alreedy RM, El-Ghareeb DK, Basry MA, Rastogi A, Kalaji HM (2015) Development of insect resistant maize plants expressing a chitinase gene from the cotton leaf worm *Spodoptera littoralis*. Sci Rep 5:18067

Ostrowski S, Dierick HA, Bejsovec A (2002) Genetic control of cuticle formation during embryonic development of *Drosophila melanogaster*. Genetics 161:171–182

Pan Y, Lü P, Wang Y, Yin L, Ma H, Ma G, Chen K, He Y (2012) *In silico* identification of novel chitinase-like proteins in the silkworm, *Bombyx mori*, genome. J Insect Sci 12:150–163

Pesch YY, Riedel D, Patil KR, Loch G, Behr M (2016) Chitinases and imaginal disc growth factors organize the extracellular matrix formation at barrier tissues in insects. Sci Rep 6:18340

Pillai C, Paul W, Sharma CP (2009) Chitin and chitosan polymers: chemistry, solubility and fiber formation. Prog Polym Sci 34:641–678

Reddy KRK, Rajam MV (2016) Targeting chitinase gene of *Helicoverpa armigera* by host-induced RNA interference confers insect resistance in tobacco and tomato. Plant Mol Biol 90:281–292

Resch Y, Blatt K, Malkus U, Fercher C, Swoboda I, Focke-Tejkl M, Chen KW, Seiberler S, Mittermann I, Lupinek C, Rodriguez-Dominguez A, Zieglmayer P, Zieglmayer R, Keller W, Krzyzanek V, Valent P, Valenta R, Vrtala S (2016) Molecular, structural and immunological characterization of Der p18, a chitinase-like house dust mite allergen. PLoS One 11:1–19

Reynolds SE (1987) The cuticle, growth and moulting in insects: the essential background to the action of acylurea insecticides. Pestic Sci 20:131–146

Royer V, Fraichard S, Bouhin H (2002) A novel putative insect chitinase with multiple catalytic domains: hormonal regulation during metamorphosis. Biochem J 366:921–928

Ruiz-Herrera J, San-Blas G (2003) Chitin synthesis as a target for antifungal drugs. Curr Drug Targets Infect Disord 3:77–91

Samuels RI, Reynolds SE (1993) Moulting fluid enzymes of the tobacco hornworm, *Manduca sexta*: timing of proteolytic and chitinolytic activity in relation to pre-ecdysial development. Arch Insect Biochem Physiol 24:33–44

Saxena IM, Brown RM Jr, Fevre M et al (1995) Multidomain architecture of beta-glycosyl transferases: implications for mechanism of action. J Bacteriol 177:1419

Shen Z, Jacobs-Lorena M (1997) Characterization of a novel gut-specific chitinase gene from the human malaria vector *Anopheles gambiae*. J Biol Chem 272:28895–28900

Soltani N, Chebira S, Delbecque J, Delachambre J (1993) Biological activity of flucycloxuron, a novel benzoylphenylurea derivative, on Tenebrio molitor: comparison with diflubenzuron and triflumuron. Experientia 49:1088–1091

Stern R, Jedrzejas MJ (2008) Carbohydrate polymers at the center of life's origins: the importance of molecular processivity. Chem Rev 108:5061–5085

Suzuki K, Taiyoji M, Sugawara N, Nikaidou N, Hernissat B, Watanabe T (1999) The third chitinase gene (ChiC) of *Serratia marcescens* 2170 and the relationship of its product to other bacterial chitinases. Biochem J 343:587–596

Tellam RL (1996) Protein motifs in filarial chitinases: an alternative view. Parasitol Today 12:291–292

Tellam RL, Eisemann C (2000) Chitin is only a minor component of the peritrophic matrix from larvae of *Lucilia cuprina*. Insect Biochem Mol Biol 30:1189–1201

Tellam RL, Vuocolo T, Johnson SE, Jarmey J, Pearson RD (2000) Insect chitin synthase. Eur J Biochem 267:6025–6043

Tetreau G, Cao X, Chen Y-R, Muthukrishnan S, Jiang H et al (2015) Overview of chitin metabolism enzymes in *Manduca sexta*: identification, domain organization, phylogenetic analysis and gene expression. Insect Biochem Mol Biol 62:114–126

Tharanathan RN, Kittur FS (2003) Chitin – the undisputed biomolecule of great potential. Crit Rev Food Sci Nutr 43:61–87

Van Leeuwen T, Demaeght P, Osborne EJ et al (2012) Population bulk segregant mapping uncovers resistance mutations and the mode of action of a chitin synthesis inhibitor in arthropods. Proc Natl Acad Sci 109:4407–4412

Veronico P, Gray L, Jones J et al (2001) Nematode chitin synthases: gene structure, expression and function in *Caenorhabditis elegans* and the plant parasitic nematode *Meloidogyne artiellia*. Mol Gen Genomics 266:28–34

Wilson TG, Cryan JR (1997) Lufenuron, a chitin synthesis inhibitor, interrupts development of *Drosophila melanogaster*. J Exp Zool 278:37–44

Wittkopp PJ, Beldade P (2009) Development and evolution of insect pigmentation: genetic mechanisms and the potential consequences of pleiotropy. Semin Cell Dev Biol (A Special Edition on Biosensors and Development of Pigment Cells and Pigment Patterns). Elsevier 20:65–71

Wu Q, Liu T, Yang Q (2013) Cloning, expression and biocharacterization of OfCht5, the chitinase from the insect *Ostrinia furnacalis*. Insect Sci 20:147–157

Yang B, Zhang M, Li L, Pu F, You W, Ke C (2015) Molecular analysis of atypical family 18 chitinase from fujian *Oyster crassostrea angulata* and its physiological role in the digestive system. PLoS One 10(6):1–13

Yang M, Wang Y, Jiang F et al (2016) miR-71 and miR-263 jointly regulate target genes chitin synthase and chitinase to control Locust molting. PLoS Genet 12:e1006257

Yeager AR, Finney NS (2004) The first direct evaluation of the two-active site mechanism for chitin synthase. J Organomet Chem 69:613–618

Zechel DL, Withers SG (2000) Glycosidase mechanisms: anatomy of a finely tuned catalyst. Acc Chem Res 33:11–18

Zhang X, Yan Zhu K (2013) Biochemical characterization of chitin synthase activity and inhibition in the African malaria mosquito, *Anopheles gambiae*. Insect Sci 20:158–166

Zhang X, Zhang J, Zhu K (2010) Chitosan/double stranded RNA nanoparticle mediated RNA interference to silence chitin synthase genes through larval feeding in the African malaria mosquito (*Anopheles gambiae*). Insect Mol Biol 19:683–693

Zhang J, Zhang X, Arakane Y, Muthukrishnan S, Kramer KJ, Ma E, Zhu KY (2011) Comparative genomic analysis of chitinase and chitinase-like genes in the African malaria mosquito (*Anopheles gambiae*). PLoS One 6:e19899

Zhang D, Chen J, Yao Q, Pan Z, Chen J, Zhang W (2012) Functional analysis of two chitinase genes during the pupation and eclosion stages of the beet armyworm *Spodoptera exigua* by RNA interference. Arch Insect Biochem Physiol 79:220–234

Zhu Q, Deng Y, Vanka P, Brown SJ, Muthukrishnan S et al (2004) Computational identification of novel chitinase-like proteins in the *Drosophila melanogaster* genome. Bioinformatics 20:161–169

Zhu Q, Arakane Y, Beeman RW, Kramer KJ, Muthukrishnan S (2008a) Characterization of recombinant chitinase-like proteins of *Drosophila melanogaster* and *Tribolium castaneum*. Insect Biochem Mol Biol 38:467–477

Zhu Q, Arakane Y, Beeman RW, Kramer KJ, Muthukrishnan S (2008b) Functional specialization among insect chitinase family genes revealed by RNA interference. Proc Natl Acad Sci 105:6650–6655

Zhu Q, Arakane Y, Banerjee D, Beeman RW, Kramer KJ, Muthukrishnan S (2008c) Domain organization and phylogenetic analysis of the chitinase-like family of proteins in three species of insects. Insect Biochem Mol Biol 38:452–466

Zhu KY, Merzendorfer H, Zhang W et al (2016) Biosynthesis, turnover, and functions of chitin in insects. Annu Rev Entomol 61:177–196

Zhuo W, Fang Y, Kong L et al (2014) Chitin synthase A: a novel epidermal development regulation gene in the larvae of *Bombyx mori*. Mol Biol Rep 41:4177–4186

Zimoch L, Hogenkamp D, Kramer K, Muthukrishnan S, Merzendorfer H (2005) Regulation of chitin synthesis in the larval midgut of *Manduca sexta*. Insect Biochem Mol Biol 35:515–527

Correlation of Insects with Forensic Sciences

15

Mian Sahib Zar and Moli Huang

Abstract

This chapter portrays the importance of insects in forensic sciences and highlights that how the insects are used as evidence in the court and how they can assist in solving crimes. Forensic science plays a key role in the investigation of crimes and terrorism. Forensic entomology is one of the emerging fields of forensic sciences which aids in legal investigation. Postmortem interval or time since death is the time elapsed from death to the discovery of the corpse. Various methods are used to estimate postmortem intervals. They include algor mortis, livor mortis, rigor mortis, and chemical and enzymatic changes. Insects also play a significant role in estimation of time since death which is a prime concern in the field of forensic medicine. Insects are considered to be the first visitors on any decomposing dead matter. Forensic entomology is a field that is highly neglected around the world so far. Much research work is required to flourish this field for the purpose of forensic investigation. The basic challenge for forensic entomologists is the identification of insects at larval stages as they all look similar. The taxonomic keys for identification at this level are still unavailable. There is a need to cover the drawbacks of morphological identification of these species especially if the adult form of insect is not available.

M.S. Zar (✉)
School of Biology and Basic Medical Sciences, Soochow University,
Dushu Lake Higher Education Town, Suzhou Industrial Park, Suzhou, Jiangsu 215123, China
e-mail: msahibzar@yahoo.com

M. Huang
School of Biology and Basic Medical Sciences, Soochow University,
Dushu Lake Higher Education Town, Suzhou Industrial Park, Suzhou, Jiangsu 215123, China

National Engineering Laboratory for Modern Silk, Soochow University,
Suzhou, Jiangsu 215123, China

D. Kumar, C. Gong (eds.), *Trends in Insect Molecular Biology and Biotechnology*, https://doi.org/10.1007/978-3-319-61343-7_15

15.1 Introduction

Forensic (Latin word; meaning "in open court" or "public") is a field of science that solves legal issues in both criminal and in civil cases (Jobling and Gill 2004). Insects are the most abundant living creatures that exist on the dry lands of earth. Though few have some aquatic adaptations as well, mainly they tend to like land masses. Insects are invertebrates of class *Insecta* belonging to phylum *Arthropoda* and kingdom *Animalia* in the domain of *Eukaryota*. Usually not classified as animals, insects are most diverse group having more than six to ten million extant species, making them more than half of all the known living organisms, though the described species are more than a million. The word "insect" comes from Latin word "*insectum*" meaning divided into segments. Their study is called entomology, having been derived from the Greek word "*entomon*" meaning "cut into two or cut in segments." General characteristics are an exoskeleton composed of chitin; three parts of the body, i.e., the head, thorax, and abdomen; three pairs of legs; compound eyes and a pair of antennae; and in some two wings as well (Triplehorn and Johnson 2004).

Insects are one tough group of living organisms which are highly adaptable and exist in nearly any habitat. In their role as part of the ecosystem, they are both beneficial and a nuisance for other organisms including humans. The beneficial roles are production of honey, wax, silk, shellac, or parts of cosmetics. Some are used as good source of protein-rich food. The nuisance factors are insects being vectors for harmful agents of many diseases of man and animals. Also the insect bites, sting, or infestation can cause serious illnesses or even death. Common examples of insects are ants, flies, mosquitoes, termites, beetles, wasps, cockroaches, lice, fleas, butterflies, etc. (Lord and Rodrigues 1989).

The development is variable, but mainly the insects develop through a process of holometabolism, i.e., going through a complete process of metamorphosis that includes four stages of oviposition (or in some viviparous species by direct laying of larvae), larval stage, pupa, and then imago or adult insect. Insects play an important role in the scavenging of the decomposed dead organic matter other than bacteria and fungi. As processors of dead animals, they consume most of the carcasses if they are uncovered, otherwise if covered the carcasses mummify. The two specialized features of smell for detection and locomotion for flight help insects to approach dead matter and consume it. The gases and body fluids including blood produce specific odors that attract many scavengers including insects. They are capable of consuming all carcasses except bones. The pattern of succession varies with seasons, habitats, and countries, but the basic pattern of succession is constant around the world (Lord and Rodrigues 1989).

Insects are first visitors from external environment for any dead matter, including human corpses. Whether humans face natural death or die under some foul play, the succession of insects is as same as in other dead matter. This leads to yet another special type of study known as "forensic entomology" which can be defined as the *study of insects succeeding dead remains to aid legal investigations*. The field includes medicolegal, urban, and pests. The sequential colonization of a corpse as part of decomposition saga and the rates of development of their offsprings are

analyzed and reported to find out the cause and manner of death as well to narrow down the search of suspect (Amendt et al. 2004).

The predictable metamorphosis of insects acts as a perfect timeline to find out how long it has been for a corpse being dead. This predictability is well utilized by forensic entomologists to measure the time since death or postmortem interval in cases of suspicious deaths where bodies are found unattended and gone through a certain stage of decomposition (Villet and Amendt 2011).

These insects also make a part of circumstantial evidence at the scene of crime whether found alive or dead, facilitating forensic scientists to rule out many aspects of crime including foul play, addiction, neglect, infestation, suicides, offense, and murders. A trained forensic scientist or a crime scene investigator should be well aware of stages of decomposition and the sequence of entomological signs on the dead body as well as at the scene of crime. So, forensic scientists, entomologists, and investigators should be well equipped to collect proper evidence, its proper handling, transport, and methods to deal with such evidence alive or dead (Lord and Rodrigues 1989).

15.2 History of Forensic Entomology

The role of insects in assisting decomposition and helping natural process of organic matter consumption has been studied in the past centuries. Many paintings, figurines, and drawings in previous literature or artwork suggest the observation of decomposition process by the artists and scientists in the past. The first forensic entomological evidence-related medicolegal case has been reported in medicolegal textbook Hsi Yuan Lu (*The Washing Away of Wrongs*) by Chinese lawyer and death investigator Sung Tz'u in the thirteenth century. The case described is of a stabbing of a man near the rice field. The investigator asked all the farmers to gather at a place and asked them to put down their sickles on the floor. All sickles seemed clean, but blowflies gathered on one of the sickle that still had invisible traces of blood. When investigated from the tool owner, he confessed to the murder.

Carl Von Linne in 1767 made observation that three flies destroyed a horse faster than a lion by producing large numbers of maggots. Then in the eighteenth and nineteenth century, French and German observed that buried bodies are consumed by insects. French physician Bergeret in 1855 published the first case report that included estimation of postmortem interval by making use of modern forensic entomology. Colonization interval estimation started in the nineteenth century as mentioned in the famous book of *La faune de cadavres* by Pierre Megnin. This method spreads to Canada and the United States and Europe giving rise to species lists and monograph publications in 1920 (Benecke 2004). Again in 1935 forensic entomology was successfully utilized in the most famous Buck Ruxton case. In 1958 a valuable study on insects and their relationships with decay rates was reported by Reed.

In the twentieth century, much work has been done on the taxonomy, and many new species have been discovered, and their life cycles have been studied in detail. Regarding the medicolegal importance of insects' presence at scene of crime and

the time of colonization, much work has been done in Europe specially. The new trend is to describe the life cycles of forensically important insects under different biogeographical environments to rule out foul play, neglect, assaults, and murders, and much success has been achieved to this end lately. Still the science of forensic entomology needs more encouragement for further researches in this area to improve our judicial and forensic system.

15.3 Medicolegal Importance of Different Insects

The use of insects in medicolegal investigations hides in the fact that they have been studied extensively with already reported life cycles that are constant for each species, so with the help of this available data, a fair and accurate prediction can be given for the sequence and time of events that lead to a crime, assault, or murder. The group of interest for forensic investigators and forensic entomologists is arthropods and so the insects. Once they infest the scene or body, they naturally start a biological clock that keeps ticking until they are found, collected, and reared to give an accurate time since colonization.

Many factors affect the study of insects especially regarding a medicolegal scene of crime. Most important factor is the environmental temperature. The colder the weather, the longer is the life cycle and vice versa. Other facts affect the life cycles too, including humidity, seasons, rainfalls, sunlight, shade, and off course not to mention the food source. The limitations are the arthropods present at the scene but advantageous as well, as the collection is easier and limited to those species.

To recognize and learn the manifestations of decomposition and to associate these with insects present at a particular stage of decomposition need knowledge of entomology and its training. The first presence of insects is in the form of eggs on the decomposing dead matter or body. It is technically the first visible stage and looks like rice-like particles on the body orifices initially. The other initial stage can be threadlike worms at same locations if some insect species has directly laid worms

Table 15.1 The faunal succession

Succession	Decomposition stage	Time of decomposition	Insects
1	Fresh	First 3 months	Flies: blowflies
2	Odor	–	Flies: blowflies and flesh flies
3	Rancid fats	3–6 months	Dermestid beetles
4	–	–	Various flies
5	Ammonia/fermentation	4–8 months	Various flies and beetles
6	–	1–12 months	Mites
7	Completely dry	1–3 years	Dermestid beetles
8	–	3+ years	Beetles

as in flesh flies. Rather than ignoring the collection of live specimen, it should be started at the very moment (Shin et al. 2015). Though the following facts shouldn't be ignored:

- The stage of decomposition of the dead remains as algor mortis, livor mortis, and rigor mortis has to be noted and properly photographed.
- Egg deposition on the body orifices or flexures has to be observed before handling of corpse.
- See if larvae are present, and if they are, they have not dispersed far from the body.
- Eggs when seen should be collected; same is true for larvae, pupae, or dead young flies at the scene of crime.
- Make sure no entomological evidence is lost, and proper supplies and equipment should be part of crime scene investigator's equipment list.

15.3.1 Estimation of Postmortem Interval

Forensic entomology should be an integral part of crime scene investigations especially in sudden and unnatural deaths. It can help to estimate the time elapsed since death particularly when more than 3 days have elapsed. The entomological time since death depends upon two important components: period of isolation and time since colonization. The period of isolation is the time of arrival of forensically

Table 15.2 Information largely helps investigators to collect insects from crime scene

Insect group	Forensic importance
Necrophagous flies Diptera: Calliphoridae (blowflies; blue and green bottle → oviparous) Sarcophagidae (flesh flies → viviparous)	Most primary and important isolatable species for forensic investigation for postmortem interval estimations
Parasites and predators of necrophagous species Coleoptera (beetles) Parasites of dipteran larva (necrophages)	Second most important group
Omnivorous species *Wasps*, *ants*, *beetles* Feed on corpse and arthropods	Adversely affect investigations by eating Necrophagous species
Adventives species Use corpse as extension of normal habitat *Collembolan*, *spiders*, *centipedes* Acari Fungi feeders	Less valuable insects

important insects after the death of a person, while the time since colonization is actually the estimated age of insects collected from the body. Blowflies are the first individual to arrive in the crime scene as they accumulate within minutes to few hours (Greenberg 1991).

The larvae of different species of insects develop at different times or rates even under similar temperature regimes. After hatching of eggs, the larvae go through three growth phases called “instars” separated by molting of larval skin. After the third instar stage, the larva goes into post-feeding stage or prepupal stage. It is also the wandering stage of post-feeding larva where the larva leaves the wet environment of flesh and body and seeks a drier place to pupate and rest. Then it goes through metamorphosis and converted into imago or adult stage of insect (Fig. 15.1). For complete developmental analysis, available temperature data nearest the crime scene along with the life cycle of that species from former research should be considered.

Various methods are used worldwide to calculate time since death by entomological evidence. Some are stages of succession, age-dependent changes in the intestinal contents, onstage invasion, developmental patterns, weights of larvae, isomegalen/isomorphen diagrams, fly eggs, from insect’s gut contents, from cuticular hydrocarbons, width, from accumulated degree days/hours (ADD/ADH), aging blowfly eggs through gene expression, ontogenetic study, effect of body length and crawling speed, larval dispersal, length of larvae, pupae, internal morphological analysis of pupae, new simulation model, differential gene expression during metamorphosis, estimation of age with 3D micro-computed tomography, and volatile organic compounds released by larvae and pupae (Sharma et al. 2015).

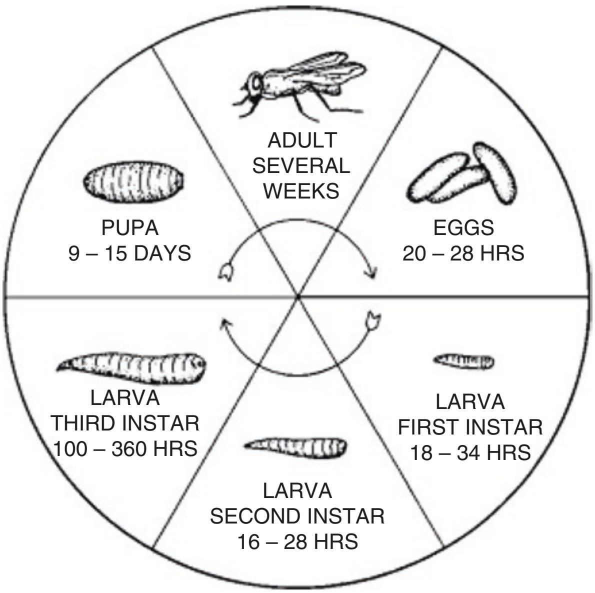


Fig. 15.1 Life cycle of insects (adapted from Krikken and Huijbregts (2001))

15.3.2 Importance in Corpse Relocation

The insects revealed the original location of crime when the body is removed from the actual place due to the absence of species of a particular place. This could help the police investigator that crime is committed somewhere else. The place where the body is found is of utmost importance for circumstantial findings as well as for collection and detection of entomological fauna. A detailed and precise description of the locality where the body is found gives an idea of the habitat to the forensic entomologists. Written notes along with photographs are necessary to assess the habitat type, terrain, vegetation, soil, and exposure of the body to sunlight or rain. The climatic conditions profoundly affect the faunal succession on the corpse. Temperature is the most important factor followed by climatic data and weather conditions like rainfall, cloud cover, wind speed, and direction. When properly collected forensically important insects prove to be a powerful tool for detection of homicide, sudden deaths, assault, and violent crimes (Varatharajan and Sen 2000).

15.3.3 Detecting the Drug Trafficking by Forensic Insects

The insects are useful source for linking the origin of drugs in drug trafficking cases (Varatharajan and Sen 2000).

15.3.4 Forensic Entomotoxicology for Detecting Controlled Substances

Insects when feed on cadaver tissues (Fig. 15.2) will also ingest and store toxicological substances consumed by the person just before death or stored in tissues for a



Fig. 15.2 Morphology of few insects found on corpses (adapted from Divya and Sathe (2015))

longer time. Successful extraction of these substances from the insects can solve cases of substance abuse, suicide, or poisoning especially when the corpse is decomposed beyond performing toxicological analysis on tissues. Though the amount of substances can be difficult to calculate to accuracy, however, qualitative results can still lead to useful information where crime scenes are concerned. The forensic insect is the source of detecting cases of narcotics when corpse is putrefied to identify. GC-MS and HPLC are techniques utilize to find toxins in insects present on corpse. So the detection of drugs in chitinized insect coins the term forensic entomotoxicology. The presence of toxicological material that causes alteration of growth pattern of arthropods also demonstrated to play key role in determining PMI (Joseph et al. 2011).

15.3.5 Trends of Insect Molecular Biology in Forensic Sciences

The fact that entomology experts are dramatically decreasing since 1990s, the identification of arthropods on morphological level is getting difficult nowadays. DNA typing since 1985 is becoming a powerful identification tool in the field of forensic sciences and medicine due to the following reasons (Jeffreys et al. 1985):

- Huge diagnostic information are obtained for crime investigation through molecular techniques as compared to older methods like blood group typing.
- DNA is present in all biological tissues except RBCs, which can be easily used for forensic investigation.
- DNA is much resistant to outside degradation than other biological molecules like proteins.
- Molecular biology allows the use of DNA for forensic investigation even if DNA is strongly degraded and broken into short pieces.

It can be useful if DNA settings of medicolegal centers are able to support forensic entomologists with DNA typing to get genetic fingerprints of insect specimen. The usual specimen is human DNA, so finding services for insect DNA typing can be unusual. The main goal nowadays is to find suitable PCR primers or sequencing sites for insect identification on species level (not on individual level). So the possible targets are all types of repetitive DNA like random amplified polymorphic DNA (RAPDs), STRs, and all mini-satellite DNA, as well as non-repetitive DNA and unique sites on mtDNA (mitochondrial DNA). Insects have noncoding mtDNA region that contains high proportions of adenine and thymine bases that are useful for DNA typing in forensic entomology. Fly mtDNA has considerable biological information that makes it easy to design PCR primers and to interpret the results of any study on new fly species. The base sequence of protein-coding genes like cytochrome oxidase subunits 1 and 2 (CO1+2) may help in identification of species (Rein 2001). The activity of flies at a crime scene can lead to artifacts (small spots) which are valuable sources of DNA typing. Sometimes those traces might be the only source of information for DNA typing (Kulstein et al. 2015). Necrophagous and hematophagous arthropods and their excretion products might also be used as a source of victim DNA to assist in identification of the crime (Campobasso et al. 2005).

Both human and nonhuman DNA profiles can be obtained from insect, as fly artifacts may contain DNA from corpses which might give valuable information in cases where a corpse had been removed (Mehus and Vaughan 2013).

15.3.6 Diversity of Diagnosis

At the scene of crime, forensic scientists, medicolegal investigators, and police officers have to cooperate to rule out foul play at crime scene as well as to reach the motive of crime and cause of death. By properly collecting forensically important evidences including the entomological evidence, not only postmortem interval can be estimated but other causes like child abuse or neglect and elderly neglect can be evaluated as well. This can lead to conviction of not only parents of neglected children or adult kids of neglected old people but also of welfare workers involved.

In a particular case of a 41-year-old physician's death, maggots were found in only one eye socket, which was unusual. A bed light (40 W light bulb) had been switched on in the bedroom. All other lights were switched off and no direct sunlight could enter the room. Maggots flee light so obviously they invaded the eye that was farther from the light source. As the mummification of the body progressed, food source restricted, so maggots moved to the eye on which light was shining (Rein 2001).

15.3.7 Study of Wound Artifacts

15.3.7.1 Lesions

It started during the nineteenth century when bite patterns of cockroaches and ants became a topic of interest. The abrasions of the skin caused by these insects were mistaken for signs of poisoning, e.g., the insect tracks are mistaken for trickling of acid down the chin and neck or bite marks of beetles sometimes resemble a gunshot wound even. In the 1930s it was reported that maggots can enter spongiosa of the long bones to reach the bone marrow by creeping through foramina nutricia. Dermestid beetles feed on dried-up corpses, while bacon and corpse beetles will cause lesions that may resemble close-range gunshot wounds.

15.3.7.2 Blood Spatter

Actual blood from the crime scene can be transferred by blowflies to produce fake blood-spatter patterns. The differentiating points from the real blood spatter can be:

- Stains have tail-to-body ratio greater than 1.
- Stains having tadpole/sperm shape.
- Stains having sperm cell-type structure not ending in a small dot.
- Stains with no distinguishing tail and body.
- Stains with wavy and irregular linear structure.
- Stains with no directional consistency with other stains that have a point of convergence at a point of origin.

15.3.8 Miscellaneous Roles of Insects

- History suggests that weapons can be identified by entomological evidence.
- Suspects can be linked to scene of crime if they are bitten by arthropods specific to crime vicinity.
- Cases of children and elderly neglect by relatives or nursing staff can be solved by blowfly larvae.
- If the dead body has been moved after death, then the faunal succession can give an idea by finding insects not belonging to that vicinity.
- Circumstantial evidence in form of hygiene aspects of crime scene as presence of maggots in food or clean rooms can be explained by linking of entomological evidence with surroundings.
- Forensic entomotoxicology is yet another field where toxic drugs can be detected from the insects that fed on dead body.
- The unusual feeding sites of maggots or beetles on the body can always give clues about lethal wounds on the body as in stab wounds.
- The time and location of death can be told with the help of insects by their bio-geographical details and weather conditions.

15.3.9 Collection of Entomological Evidence

Ideally a forensic entomologist should be part of crime scene unit. The challenges of the crime scene can be unpredictable and never ideal. So, the crime scene mobile unit should be well equipped to collect all critical data including entomological evidence. Samples should be collected from on, in, and beneath the corpse. Collect adult flies with hand nets. After collection samples must be subdivided into preserved and alive samples to be reared to adult insects. Immature insects or larvae are reared on beef liver or small musculature pieces from the corpse. The following table adapted from Byrd (2010) shows supplies and equipment required for collecting entomological data:

Table 15.3 Equipment and supplies for collection of insects larvae

Item	Reason
Aerial or sweep net: 15–18 in. with 24–36 in. collapsible metal handles	For collecting flies around area of the dead body
Collection vials: screw cap-type vials with neoprene cap inserts (4-dram size) or wide mouth jar. Jars filled with ½ in. of powdered plaster, paper towel, or cotton balls to absorb ethyl acetate	Kill jars for collecting maggots
Featherweight (or light touch) forceps: these are commercially available from most biological supply houses. Using normal forceps is risky because, if too much force is used, larvae can be killed	For collecting without damaging delicate and soft fly larvae

Table 15.3 (continued)

Item	Reason
Plastic "yogurt" or "bait" containers: 16–64 oz. in size	For collecting and shipping larvae
Aluminum foil	To hold live larvae and food source during shipment (precut potato wrappers work well)
Vermiculite (or dirt from scene)	For filling the bottom of the larval containers to allow for migration and to absorb excess fluids during shipment
Plastic specimen containers: 4–8 oz. size	Additional collection containers
Paper labels (adhesive and nonadhesive, heavy bond paper)	*Nonadhesive*: used to label inside of preserved and live specimen containers
	Adhesive: for labeling outside of containers
Graphite pencil	For making labels (preservation fluids will cause ink to smear)
Small hand trowel or garden spade	For sampling soil and digging for migrating larvae or pupae in outdoor death scenes
Thermometer: digital	Used for taking temperature of area around the body and maggot mass
Photographic equipment (including scales)	Need to capture forensically relevant photographs: establishing mid-range and close-ups (of insects)
Chemicals: ethyl acetate, ethanol, KAA	Used to kill insects
Paper towel	For cleaning jars, cleaning utensils, and drying hands after disinfecting
Disposable gloves	For personal protection
Sifting screens	Used to process soil samples for collecting insects and insect artifacts
Form for death scene investigation	For record keeping
Shipping containers: styrofoam containers with lids are best because they are insulated. Corrugated cardboard boxes are cheap and commonly used	Used to ship collected specimens to appropriate experts

After collecting entomological evidence, they are taken to medical examiner's office, which ensures its delivery to forensic entomologist who works with the same team to collaborate with results. Sample containers are properly labeled like:

1. Data collected
2. Time collected
3. Location of remains
4. Area of body infested
5. Name, address, and telephone number of collector

In cases of neglect or sexual assaults, the phenomenon of myiasis exists side by side of corpse infestation. In cases of geriatric neglect, the bedsores and injuries and, in cases of child sexual assaults, perianal and genital areas should be carefully inspected to find evidence of myiasis. The entomological evidence can survive extreme weather conditions such as even fire. Intense fires may not destroy entomological or forensic evidence as fires do not prevent insect colonization or the capacity to determine postmortem intervals, though fires speed up colonization by between 1 and 4 days (Gomes et al. 2006).

15.3.10 Rules for Forensic Entomologist in Crime Scene

1. Take clear and good photographs of areas of collection of insects. There can be a rapid change in the decomposed area infested. The bite marks of mites should be marked on living persons.
2. Always take photographs without using flash because the maggots will be "flashed out" or appear "just white nothing" especially on digital photos.
3. A metric and inch scale should be used for every single picture.
4. One spoon full of insects from at least three different locations on dead corpse and crime scene must be collected in three different and properly labeled jars.
5. Put half of insects in 98% ethanol. Do not use hand cleaning alcohol or formalin. Killed insects can be stored frozen with or without alcohol.
6. Kill the insects in boiling water (tea water) before storing in alcohol.
7. Take living insects and refrigerate (do not freeze). They should be covered with a fabric so they can breathe. Transport insects to a biologist within 1–2 days. Separate white from brown larvae and brown from adults or post-feeding adults.
8. Properly label insect evidence with location, time, date, and initials.
9. If any query arises at the crime scene regarding entomological evidence, call an entomologist.
10. Identification of infesting species should be done by an experienced entomologist by using proper keys. Still much identification is carried out by determination of third instar larvae of unknown species by characteristics of their mouth parts (Benecke 2004).

15.4 Future Perspectives of Forensic Entomology

Forensic entomology is an emerging field in which the insects are used as evidence to determine the death time of the corpse. It has become the most important tool in crime investigation. Unfortunately this felid is still poorly understood due to the lack of expertise, resources, knowledge, and awareness. There is need to incorporate this area in the curriculum of universities and forensic research institutes as an important tool to have insight in forensic investigation of cadavers found in the crime scene. Much research work on forensic entomology is required to flourish this field for the purpose of forensic investigation.

Conclusions

This chapter describes the correlation of insects with forensic science and emphasizes that how the insects were used as evidence in the court and how they can assist in solving crimes. In addition, it describes the history, habitat, and key roles of insects in forensic sciences. Further it highlights the need and importance of forensic entomology in crime investigation.

References

Amendt J, Krettek R, Zehner R (2004) Forensic entomology. Nature Science 91:51–65

Benecke M (2004) Forensic entomology: arthropods and corpses. In: Tsokos M (ed) Forensic pathology reviews, vol II. Humana, Totowa, NJ, pp 207–240

Byrd JH, Castner JL (2010) Forensic Entomology: The Utility of Arthropods in Legal Investigations. 2nd Edition. CRC Press, Boca Raton, 681

Campobasso CP et al (2005) Forensic genetic analysis of insect gut contents. Am J Forensic Med Pathol 26(2):161–165

Divya JK, Sathe TV (2015) Diversity occurrence and development of forensic insects in Dog canis domesticus L Carcass from Kolhapur India. Int J Pharm Bio Sci 6:498–506

Gomes L, Godoy WAC, Von Zuben CJ (2006) A review of postfeeding larval dispersal in blowflies: implications for forensic entomology. Naturwissenschaften 93(5):207–215

Greenberg B (1991) Flies as forensic indicators. J Med Entomol 28(5):565–577

Jeffreys AJ, Wilson V, Thein SL (1985) Individual-specific 'fingerprints' of human DNA. Nature 316(6023):76–79

Jobling MA, Gill P (2004) Encoded evidence: DNA in forensic analysis. Nat Rev Genet 5(10):739–751

Joseph I et al (2011) The use of insects in forensic investigations: an overview on the scope of forensic entomology. J Forensic Dent Sci 3(2):89–91

Krikken J, Huijbregts J (2001) Insects as forensic informants: the Dutch experience and procedure. Proc Exp Appl Entomol 12:159–164

Kulstein G, Amendt J, Zehner R (2015) Blow fly artifacts from blood and putrefaction fluid on various surfaces: a source for forensic STR typing. Entomol Exp Appl 157(3):255–262

Lord WD, Rodrigues WC (1989) Forensic entomology: the use of insects in the investigation of homicide and untimely death

Mehus JO, Vaughan JA (2013) Molecular identification of vertebrate and hemoparasite DNA within mosquito blood meals from eastern North Dakota. Vector Borne Zoonotic Dis 13(11):818–824

Rein BM (2001) Purely unilateral occurrence of blowfly maggots in the face of a decomposing body. Arch Kriminol 208:182–185

Sharma R, Kumar Garg R, Gaur JR (2015) Various methods for the estimation of the post mortem interval from Calliphoridae: a review. Egypt J Forensic Sci 5(1):1–12

Shin SE et al (2015) The first survey of forensically important entomofauna collected from medicolegal autopsies in South Korea. Biomed Res Int 2015:6

Triplehorn CA, Johnson NF (2004) Borror and DeLong's introduction to the study of insects. Thomson Brooks/Cole, Belmont, p 888

Varatharajan R, Sen A (2000) Role of entomology in forensic sciences. Curr Sci 78(5):544–545

Villet MH, Amendt J (2011) Advances in entomological methods for death time estimation. In: Turk EE (ed) Forensic pathology reviews. Humana, New York, 213–217s

16

Nanoparticles as Precious Stones in the Crown of Modern Molecular Biology

M. Rajesh Kumar and P. Joice Sophia

Abstract

The interdisciplinary field of research on biosystems at the nanoscale involving physical sciences, molecular engineering, biology, biotechnology, and medicine supplements the knowledge of synthesizing new drugs, targeted delivery, regenerative medicine, and neuromorphic engineering forms the booming research in the present society. The present chapter deals with the role of nanoparticles in modern molecular biology. This is an interesting area of research that creates great impact on the healthcare of the society. The prime focus is to give the reader a historic background of nanomaterial application in biology and medicine. We have also provided the overview of most recent developments in this field leading to discussion of hard road to commercialization.

16.1 Introduction

Every person has been exposed to nanometer-sized foreign particles; we inhale them with every breath and consume them with every drink. In truth, every organism on Earth continuously encounters nanometer-sized entities (Rizzello et al. 2011; Pandey et al. 2008; Bennett-Woods 2006). Very small particles, so-called nanoparticles, have the ability to enter, translocate within, and damage living

M. Rajesh Kumar (✉)
College of Chemistry, Chemical Engineering and Material Science, Soochow University, Suzhou 215123, China
e-mail: rajeshkumar_vgm@yahoo.com

P. Joice Sophia
Department of Nanoscience and Technology, Bharathiar University, Coimbatore 641046, India
e-mail: joicesophia@gmail.com

D. Kumar, C. Gong (eds.), *Trends in Insect Molecular Biology and Biotechnology*, https://doi.org/10.1007/978-3-319-61343-7_16

organisms (Elsaesser and Howard 2012; Prabhu and Poulose 2012). This ability results primarily from their small size thereby allowing them to penetrate physiological barriers and travel within the circulatory systems of a host (Panessa-Warren et al. 2006; Li et al. 2010; Chen et al. 2016). The vast majority causes little ill effect and goes unnoticed, but occasionally an intruder will cause appreciable harm to the organism. The most advanced of the toxic intruders are viruses, composed of nucleic acid-based structures that allow them not only to interfere with biological systems but also in parasitically exploit cellular processes to replicate themselves (Ravindran et al. 2016; Franzen and Lommel 2009). A growing number of recent studies show, however, that nano- and microorganisms might play a vital role in many chronic diseases where infection pathogens have not been suspected, diseases that were previously attributed only to genetic factors and lifestyle (He and Shi 2009; Zheng et al. 2016). These diseases include leukemia (caused by viruses from the retrovirus and herpesvirus families) (Nisole et al. 2005; Walther and Stein 2000; Jarrett 2006), cervical cancer (*Papillomavirus*) (DiMaio and Liao 2006; Muñoz et al. 2003; Bosch et al. 1995), liver cancer (hepatitis virus), gastric ulcer (*Helicobacter pylori*) (Kusters et al. 2006; Blaser et al. 1995; Tomb et al. 1997), nasopharyngeal cancer (*Epstein-Barr virus*) (Zur Hausen et al. 1970; Lo et al. 1999; Burgos 2005), kidney stones (nanobacteria) (Çiftçioglu et al. 1999; Kramer et al. 2000; Kajander and Ciftcioglu 1998), severe acquired respiratory syndrome SARS (coronavirus) (Peiris et al. 2003; Rota et al. 2003; Marra et al. 2003; Kahn 2006), heart disease (*Chlamydia pneumonia*) (Patel et al. 1995; Danesh et al. 1997; Bachmaier et al. 1999; Kol and Santini 2004), juvenile diabetes (*Coxsackievirus*) (Atkinson et al. 1994; Horwitz et al. 1998; Fohlman and Friman 1993), Alzheimer's disease (*Chlamydia pneumoniae*) (Balin et al. 1998; Hammond et al. 2010; Itzhaki et al. 2004), pediatric obsessive-compulsive disorder (*Streptococcal* bacteria) (Swedo et al. 1998; Mell et al. 2005; Lynch et al. 2006), psychotic disorders (bornavirus) (Nunes et al. 2008; Miranda et al. 2006), and prion diseases such as mad cow disease (proteins-prions) (Chesebro 1998; Legname et al. 2004; Janka and Maldarelli 2004).

Nanotechnology is the manipulation of matter at the scale of 1–100 nanometers. Using nanotechnology, we can control molecules at an atomic level and create materials with unique properties. A nanometer is 10^{-9} (a billionth) of a meter. The prefix nano has the Greek meaning of dwarf. As a reference point, a hair is approximately 100,000 nanometers. A red blood cell is approximately 10,000 nanometers. Fundamentally the properties of materials can be changed by nanotechnology. We can arrange molecules in a way that they do not normally occur in nature. The material strength along with electronic and optical properties of materials could be altered with the aid of nanotechnology.

16.1.1 State of the Art

Molecular biology explores cells and their characteristics, parts, and chemical processes which pay special attention to how molecules control cell's activities and growth (Crick 1970). Looking at the molecular machinery of life that began in the

early 1930s, but truly modern molecular biology emerged with the uncovering of the structure of DNA in the 1960s (Meyer 2003; Morange and Cobb 2000). As a science that studies interactions between the molecular components that carry out various biological processes in living cells, an important idea in molecular biology states that information flow in organisms follows a one-way street: genes are transcribed into RNA, and RNA is translated into proteins. The molecular components make up biochemical pathways that provide the cells with energy, facilitate processing "messages" from outside the cell itself, generate new proteins, and replicate the cellular DNA genome. For example, molecular biologists study how proteins interact with RNA during "translation" (the biosynthesis of new proteins), the molecular mechanism behind DNA replication, and how genes are turned on and off, a process called "transcription" (Lodish et al. 1995).

The birth and development of molecular biology were driven by the collaborative efforts of physicists, chemists, and biologists. As mentioned, modern molecular biology emerged with the discovery of the double helix structure of DNA. The 1962 Nobel Prize in Physiology or Medicine was awarded jointly to Francis H. Crick, James D. Watson, and Maurice H. F. Wilkins "for their discoveries concerning the molecular structure of nucleic acids and its significance for information transfer in living material" (Palenik et al. 2003; Scott and Thompson 2011). Advances and discoveries in molecular biology continue to make major contributions to medical research and drug development (Lipinski and Hopkins 2004).

DNA sequencing and synthesis are two sides of the same coin, the "read" and "write" functions of genetic material (Shendure and Ji 2008; Chan 2005). This field and its requisite technology took off in the 1990s with the Human Genome Project's effort to sequence billions of bases to unlock a new era of genetically informed medicine (Sawicki et al. 1993; Venter et al. 2001). The resulting science is still a work in progress – it turns out the genetic code a more complicated one than anticipated – but the technologies and companies helped it spawn are an impressive legacy. The Integrated DNA Technologies (IDT) got its start during the Human Genome Project, as it produced single nucleotides (the As, Ts, Cs, and Gs that comprise the genetic code) and short oligonucleotide chains (or "oligos") to facilitate a massive sequencing effort around the world (Burns et al. 1996). Of course, sequencing technology has advanced dramatically in the intervening decades, but "you still need oligos to do the sequencing," explains Jerry Steele, IDT's Director of Marketing, "especially in the next generation sequencing space, sequencing and DNA synthesis go hand in hand." The current sequencing method of choice is Illumina, a process that frequently returns millions of bases of DNA sequence by reading distinct stepwise fluorescent signals associated with each base in a massively parallel array (Schadt et al. 2010; Nakamura et al. 2011). To distinguish genetic material from different samples (a few hundred are often run on the same plate), scientists label each sample's DNA extract with a distinct barcode (Hebert et al. 2003). With each barcode comprised of about ten nucleotides, the demand for synthetic DNA chains in the sequencing process is substantial (deWaard et al. 2008).

Unlike other biotech companies prioritizing longer constructs or gene variants, IDT specializes in relatively short oligos. These chains are used not only in Illumina

barcoding but also as primers, consistent patches of sequence that may border unknown regions and facilitate polymerase chain reaction (PCR)-based amplification (Bhargava et al. 2013). Both techniques – "next-generation" Illumina sequencing and primer-based amplification – are staples of any self-respecting applied or research-based microbiology laboratory, as they allow researchers to identify constituent organisms or confirm a gene's presence. With such short sequences, a single nucleotide discrepancy could mean the difference between two Illumina samples from opposite ends of the world or between a gene native to the *Firmicutes* or the *Proteobacteria*. It's a small margin for error, "so every base better be right," explains Steele. "As we've grown, it's just a matter of maintaining that consistency on a larger scale." In the spirit of not fixing something that needs no repairs, IDT shipped an entire fabrication room from its headquarters in Coralville, Iowa, to Belgium when that facility was being built.

Fundamental as they are to modern biology, oligos are used every day in thousands of laboratories around the world, often in innovative ways that the company itself may not have predicted. "The things that people are doing with DNA are really inspiring," noted Steele. One of his favorite cases involves low-impact prenatal tests: rather than a painful and invasive amniocentesis, "we've discovered that now because of sequencing, we can see the baby's DNA in a blood draw from the mother." Improved sequencing fidelity and throughput are expanding the resolution of the technique, and Steele envisions scientists using next-generation sequencing to detect cancer cells from the bloodstream as an early diagnostic tool. "Biology is really leaving the lab and coming into the real world," Steele explains, "and it's going to improve a lot of lives."

16.1.2 Nanomaterials in Modern Molecular Biology

The interdisciplinary field of research on biosystems at the nanoscale involving physical sciences, molecular engineering, biology, biotechnology, and medicine supplements knowledge of the synthesis of new drugs and their targeted delivery, regenerative medicine, neuromorphic engineering, and developing a sustainable environment (Roco 2003). The diagnostics for early cancer detection have been made successful by the dendritic polymers, multicolor quantum dots, nanocarriers, and diagnostic markers (Ferrari 2005; Smith et al. 2006; Wolinsky and Grinstaff 2008; Majoros et al. 2008; Peer et al. 2007; Visintin et al. 2008). Gold nanoparticles play a major role in cancer therapeutics owing to its enhanced surface plasmon resonance (Huang et al. 2007; Kah et al. 2007). DNA delivery into cell applications employs silica nanoparticles (Roy et al. 2005; Kneuer et al. 2000; Slowing et al. 2008; Zhu et al. 2002). Another interesting area of research is to realize biocompatible implants for replacing damaged or worn body parts with improved tissue engineering at the nanoscale and extend its arms for building up bioartificial organs (McIntire 2002; Mendonça et al. 2008; Variola et al. 2009), nano-hydroxyapatite being a reliable candidate for bioimplants due to its excellent biocompatibility (Zhou and Lee 2011; Wang et al. 2012). Nanobiological devices

and biocompatible electronic systems for detection and control, implants of wireless systems, neuroprostheses, and parts of the neural system form the part of molecular biology applications (PROKOP 2001; Gillies et al. 2002; Mercanzini et al. 2010; Tokárová et al. 2013). There is an interesting classification of nanotechnology in two classes, "wet" nanotechnology (living biosystems) and "dry" nanotechnology. The systematic approaches are important in engineering man-made objects at the nanoscale for integrating them into large-scale structures which are similar to that of nature (Sarikaya et al. 2003; Alivisatos 2004). Interestingly, this booming field is also known as biomimicry or bionics where researchers get the idea from nature by mimicking them to be explored in various applications (Chakrabarti and Shu 2010; Quinn and Gaughran 2010). Few examples of such concepts can be lotus effect, pearl effect, Gecko effect, butterfly wings, Namib desert beetle, spider web, and so on (Bhushan 2009; Pereira et al. 2015). These biomimetic ideas were adopted for the successful fabrication of Velcro, self-cleaning tiles, desert fog harvesting, water filters, adhesives, nanopaints, etc. (Bhushan and Jung 2011; Vierra 2011; Ivanić et al. 2015; Diamanti and Pedeferri 2015; Weiler and Goel 2015). Some of the companies that are involved

Table 16.1 Examples of companies commercializing nanomaterials for bio- and medical applications

Company	Major area of activity	Technology
Advectus Life Sciences Inc.	Drug delivery	Polymeric nanoparticles engineered to carry antitumor drug across the blood-brain barrier
Alnis Biosciences, Inc.	Biopharmaceutical	Biodegradable polymeric nanoparticles for drug delivery
Argonide	Membrane filtration	Nanoporous ceramic materials for endotoxin filtration, orthopedic and dental implants, and DNA and protein separation
BASF	Toothpaste	Hydroxyapatite nanoparticles seem to improve dental surface
Biophan Technologies, Inc.	MRI shielding	Nanomagnetic/carbon composite materials to shield medical devices from RF fields
Capsulution NanoScience AG	Pharmaceutical coatings to improve solubility of drugs	Layer-by-layer polyelectrolyte coatings, 8–50 nm
Dynal Biotech		Magnetic beads
Eiffel Technologies	Drug delivery	Reducing size of the drug particles to 50–100 nm
EnviroSystems, Inc.	Surface disinfectant	Nanoemulsions
Evident Technologies	Luminescent biomarkers	Semiconductor quantum dots with amine or carboxyl groups on the surface, emission from 350 to 2500 nm
Immunicon	Tracking and separation of different cell types	Magnetic core surrounded by a polymeric layer coated with antibodies for capturing cells

(continued)

Table 16.1 (continued)

Company	Major area of activity	Technology
KES Science and Technology, Inc.	Airocide filters	Nano-TiO_2 to destroy airborne pathogens
NanoBio Corporation	Pharmaceutical	Antimicrobial nanoemulsions
NanoCarrier Co., Ltd	Drug delivery	Micellar nanoparticles for encapsulation of drugs, proteins, DNA
NanoPharm AG	Drug delivery	Polybutylcyanoacrylate nanoparticles are coated with drugs and then with surfactant and can go across the blood-brain barrier
Nanoplex Technologies, Inc.	Nanobarcodes for bioanalysis	
Nanoprobes, Inc.	Gold nanoparticles for biological markers	Gold nanoparticles bioconjugate for TEM and/or fluorescent microscopy
Nanoshpere, Inc.	Gold biomarkers	DNA barcode attached to each nanoprobe for identification purposes; PCR is used to amplify the signal and also catalytic silver deposition to amplify the signal using surface plasmon resonance
NanoMed Pharmaceutical, Inc.	Drug delivery	Nanoparticles for drug delivery
Oxonica Ltd	Sunscreens	Doped transparent nanoparticles to effectively absorb harmful UV and convert it into heat
pSivida Ltd	Tissue engineering, implants, drug and gene delivery, biofiltration	Exploiting material properties of nanostructured porous silicone
Smith & Nephew	Acticoat bandages	Nanocrystal silver is highly toxic to pathogens
Quantum Dot Corporation	Luminescent biomarkers	Bioconjugated semiconductor quantum dots

in the development and commercialisation of nanomaterials in biological and medical applications are listed below (Table 16.1) (Salata 2004).

16.1.3 Nanoparticles Existing in Biology

Hybrid bio-nanomaterials can also be applied to build novel electronic, optoelectronic, and memory devices (Yan et al. 2003). Self-assembling nanostructure-based DNA molecules obtained by bottom-up nanofabrication show interesting applications. The main focus is firstly to give the reader a historic prospective of nanomaterial application to biology and medicine, secondly to try to overview the most recent developments in this field, and finally to discuss the hard road to commercialization.

16.1.3.1 Nanomedicine

Nanomedicine is a field of medical science employing nanotechnology whose applications are increasing more and more, thanks to nanorobots and biological machines which become very useful tools to develop this area of knowledge (Andrew 2000; Wagner et al. 2006; Riehemann et al. 2009). Nanomedicine is the application of nanotechnology in different areas of medicine and biology (Andrew 2000). Nanomedicine ranges from the biomedical applications of nanomaterials and biological devices to nanoelectronic biosensors and even possible future applications of molecular nanotechnology such as biological machines (Nie 2010; Vinogradov and Wei 2012; Jain and Stylianopoulos 2010). Current problems for nanomedicine involve understanding the issues related to toxicity and environmental impact of nanoscale materials (Nel et al. 2006; Hardman 2006; Dreher 2004; Colvin 2003; Jia et al. 2005). The size of nanomaterials is similar to that of most biological molecules and structures; therefore, nanomaterials can be useful for both in vivo and in vitro biomedical research and applications (Salata 2004). More than just an extension of "molecular medicine," nanomedicine will employ molecular machine systems to address medical problems and will use molecular knowledge to maintain and improve human health at the molecular scale (Freitas 2002; Freitas 2005; Kostarelos 2006; Bogunia-Kubik and Sugisaka 2002). Nanomedicine will have extraordinary and far-reaching implications for the medical profession, in the definition of disease and in the diagnosis and treatment of medical conditions including aging, and ultimately for the improvement and extension of natural human biological structure and function.

16.1.3.2 Nanoinsecticide

An insecticide is a substance used to kill insects. They include ovicides and larvicides used against insect eggs and larvae, respectively. Insecticides are used in agriculture, medicine, industry, and by consumers. Insecticides are claimed to be a major factor behind the increase in agricultural twentieth century's productivity (Perry et al. 2013). Nearly all insecticides have the potential to significantly alter ecosystems; many are toxic to humans; some concentrate along the food chain.

16.2 Classification of Nanostructures

Nanostructures are classified based on the number of dimensions, which are not confined to the nanoscale range (<100 nm) (Kelsall et al. 2005):

- Zero dimensional (0D)
- One dimensional (1D)
- Two dimensional (2D)
- Three dimensional (3D)

16.2.1 Zero Dimensional (0D)

Materials wherein all the dimensions are measured within the nanoscale (no dimensions, or 0-D, are larger than 100 nm). The most common representation of zero-dimensional nanomaterials is nanoparticles or quantum dots. Nanoparticles or quantum dots can be amorphous or crystalline, single crystalline or polycrystalline, metallic, ceramic, or polymeric, and composed of single or multi-chemical elements; exhibit various shapes and forms; and exist individually or are incorporated in a matrix.

16.2.2 One Dimensional (1-D)

One dimension is outside the nanoscale leading to needlelike-shaped nanomaterials. One-dimensional materials include nanotubes, nanorods, and nanowires. One-dimensional nanomaterials can be amorphous or crystalline; metallic, ceramic, or polymeric; single crystalline or polycrystalline; chemically pure or impure; standalone materials; or embedded in within another medium.

16.2.3 Two Dimensional (2-D)

In 2-D nanostructures, two of the dimensions are not confined in the nanoscale. Two-dimensional nanomaterials exhibit platelike shapes. Two-dimensional nanomaterials include nanofilms, nanolayers, and nanocoatings. Two-dimensional nanomaterials can be amorphous or crystalline, metallic, ceramic, or polymeric, deposited on a substrate, integrated in a surrounding matrix material, made up of various chemical compositions, and used as a single layer or as multilayer structures.

16.2.4 Three Dimensional (3-D)

Bulk nanomaterials are materials that are not confined to the nanoscale in any dimension and are known as 3-D nanostructures. These materials are thus characterized by having three arbitrarily dimensions above 100 nm. Materials possess a nanocrystalline structure or involve the presence of features at the nanoscale. In terms of nanocrystalline structure, bulk nanomaterials can be composed of a multiple arrangement of nanosize crystals, most typically in different orientations. With respect to the presence of features at the nanoscale, 3-D nanomaterials can contain dispersions of nanoparticles, bundles of nanowires, and nanotubes as well as multinanolayers.

The following table shows a list of nanomedical technologies (Freitasjr 2005) (Table 16.2).

Table 16.2 Nanomedicine technologies

Raw nanomaterials	Cell simulations and cell diagnostics	Biological research
Nanoparticle coatings	Cell chips	Nanobiology
Nanocrystalline materials	Cell simulators	Nanoscience in life sciences
Artificial binding sites	*DNA manipulation, sequencing, diagnostics*	*Drug delivery*
Artificial antibodies	Genetic testing	Drug discovery biopharmaceuticals
Artificial enzymes	DNA microarrays	Drug encapsulation
Artificial receptors	Ultrafast DNA sequencing	Drug delivery
Molecular imprinted polymers	DNA manipulation and control	Smart drugs
Nanostructured materials	*Tools and diagnostics*	*Biotechnology, biorobotics, and nanorobots*
Cyclic peptides	Bacterial detection systems	Biological viral therapy
Dendrimers	Biochips	Virus-based hybrids
Detoxification agents	Biomolecular imaging	Stem cells and cloning
Fullerenes	Biosensors and biodetection	Tissue engineering
Functional drug carriers	Diagnostic and defense applications	Artificial organs
MRI scanning	Endoscopic robots and microscopes	Nanobiotechnology
Nanobarcodes	Fullerene-based sensors	Biorobotics and biobots
Nanoemulsions	Cellular imaging	DNA-based devices and nanorobots
Nanofibers	Lab on a chip	Diamond-based nanorobots
Nanoparticles	Monitoring	Cell repair devices
Nanoshells	Nanosensors	
Carbon nanotubes	Point of care diagnostics	
Noncarbon nanotubes	Protein microarrays	
Quantum dots	Scanning probe microscopy	

16.3 Fabrication Methods of Nanoparticles

The present research is more focused on green synthesis of nanoparticles without any toxic chemicals by means of reduction mechanism using plants, vegetables, and fruits (Disci-Zayed 2016; Iravani 2011). Different methods are available in the synthesis of nanoparticles. All these techniques fall into one of the three categories:

1. Solid-state synthesis of nanoparticles
2. Vapor-phase synthesis of nanoparticles
3. Soft chemical methods

16.3.1 Solid-State Synthesis of Nanoparticles

Solid-state synthesis generally involves a heat treatment step in order to achieve the desired crystal structure, which is followed by media milling (Cao 2004). While it is generally believed that it is difficult for the lower limit of the average particle size to be much below 100 nm, recent innovations by established companies in the industry may prove otherwise. In particular, the Netzsch LMZ-25 ZETA II System and the Dyno-Mill ECM may push the envelope on what mechanical attrition can do to reduce the particle size.

Judging by the contents of publications, the scientific community has not shown much enthusiasm for mechanical attrition processes for nanoparticle synthesis, perhaps due to issues pertaining to impurity pickup, lack of control on the particle size distribution, and inability to tailor precisely the shape and size of particles in the 10–30 nm range as well as the surface characteristics. Nonetheless, in several instances a modified version of mechanical attrition has been used to synthesize oxide nanoparticles. Dry milling was used to induce chemical reactions through ball-powder collisions that resulted in forming nanoparticles within a salt matrix. Particle agglomeration was minimized by the salt matrix, which then was removed by a simple washing procedure.

16.3.2 Vapor-Phase Synthesis of Nanoparticles

Gas condensation, as a technique for producing nanoparticles, refers to the formation of nanoparticles in the gas phase, i.e., condensing atoms and molecules in the vapor phase (Swihart 2003). Oddly enough, it had been practiced in the industry long before it became the subject of research in institutions worldwide. For example, Cabot Corporation in the United States and Degussa in Germany have been using atmospheric flame reactors for decades to produce megatons of such diverse nanoparticles as carbon black (used in tires and inks), silicon dioxide (used in myriad applications including additives in coffee creamers and polymers), and titanium dioxide (used in scores of applications including UV-protecting gels). The generic process involves hydrolysis of gaseous metallic chlorides under the influence of water, which develops during the oxyhydrogen reaction and which in turn leads to a high-temperature reaction zone.

16.3.3 Soft Chemical Method

Soft chemical synthesis is a method where the intended product is synthesized through moderate chemical reactions under temperatures ranging from room temperature to several hundred degrees Celsius (Bernardi et al. 2009). Compared to traditional physical synthesis routes or high-temperature ceramic synthesis routes, soft chemical methods have the following advantages:

- Low temperature, therefore low energy consumption
- Less hazardous, less waste, more environmentally benign
- Size and shape controllable products

Most soft chemical methods are carried out in liquid media, based on the reactants dissolving, diffusion, and crystallization process. Therefore, soft chemical methods have other characteristics including:

- Reactants can be completely mixed to form a uniform product phase.
- Products are growing in an open and free environment; therefore, its natural morphology will be easily seen.

Every method has its limitation. It is very difficult to synthesize certain chemicals, for example, solid nitride, by soft chemical methods. However, as environmental protection has become a larger issue, soft chemistry, which is also called "green chemistry," has quietly evolved into a major phenomenon. The soft chemical method is further classified as follows:

1. Chemical precipitation method
2. Microemulsion technique
3. Colloidal chemical method
4. Chemical synthesis method
5. Organic method
6. Sol-gel method
7. Ion implantation method

The microemulsion technique includes W/O microemulsion (reversed micelles) and O/W microemulsion. Preparation of nanoparticles of metal sulfides usually by reversed micelles, which are thermodynamically stable mixtures of four components: surfactant, co-surfactant, organic solvent, and water. Precipitation is one procedure to form nanoparticles by reversed micelles. In this method two reversed micelles containing the anionic and cationic surfactants are mixed.

The colloidal chemical method is also an important method to produce nanoparticles. The principle of colloidal method is that the solutions of the different ions are mixed under controlled temperature and pressure to form insoluble precipitates. The solvent could be water or alcohol, the precipitation anions are usually H_2S, and the prepared nanoparticles are quite transparent. Sol-gel is a colloidal suspension that can be gelled to form a solid. Sol is a colloidal system with solid characteristic; its particle size usually ranges from 1 to 1000 nm. Gel is a colloidal system with liquid characteristics. The dispersed matter in gel usually forms a framework which is filled with gas or liquid, and the percentage of dispersed phase is low at 1–3%. The gel will be dried or heated to obtain product.

16.4 Applications

A list of some of the applications of nanomaterials to biology or medicine is given below:

- Fluorescent biological labels (Bruchez et al. 1998; Chan and Nie 1998; Wang et al. 2002)
- Drug and gene delivery (Mah et al. 2000; Pantarotto et al. 2003)

- Biodetection of pathogens (Edelstein et al. 2000)
- Detection of proteins (Nam et al. 2003)
- Tissue engineering (Ma et al. 2003; De La Isla et al. 2003)
- Tumor destruction via heating (hyperthermia) (Shinkai et al. 1999)
- Separation and purification of biological molecules and cells (Molday and Mackenzie 1982)
- MRI contrast enhancement (Weissleder et al. 1990)
- Phagokinetic studies (Parak et al. 2002)

16.4.1 Fluorescent Biological Labels

Fluorescent labeling is known for its nondestructive nature and high sensitivity. This has made it one of the most widely used methods for labeling and tracking biomolecules (Sahoo 2012). Fluorescent labels can be hybridized to mRNA to help visualize interaction and activity, such as mRNA localization. An antisense strand labeled with the fluorescent probe is attached to a single mRNA strand and can then be viewed during cell development to see the movement of mRNA within the cell (Weil et al. 2010). Advantages of these labels include a smaller size with more variety in color. They can be used to tag proteins of interest more selectively by various methods including chemical recognition-based labeling, such as utilizing metal-chelating peptide tags, and biological recognition-based labeling utilizing enzymatic reactions (Jung et al. 2013). However, despite their wide array of excitation and emission wavelengths as well as better stability, synthetic probes tend to be toxic to the cell and so are not generally used in cell imaging studies. Silica nanoparticles and other different nanoparticles were widely employed in near-infrared-to-visible upconversion fluorescent applications for imaging and targeted delivery in cancer treatment (Jiang et al. 2009; Taton et al. 2000; Ow et al. 2005).

16.4.2 Drug and Gene Delivery

Drug delivery systems are engineered technologies for the targeted delivery and/or controlled release of therapeutic agents (Allen and Cullis 2004). Drugs have long been used to improve health and extend lives. The practice of drug delivery has changed dramatically in the past few decades, and even greater changes are anticipated in the near future. Biomedical engineers have contributed substantially to our understanding of the physiological barriers to efficient drug delivery, such as transport in the circulatory system and drug movement through cells and tissues; they have also contributed to the development of several new modes of drug delivery that have entered clinical practice. Yet, with all of this progress, many drugs, even those discovered using the most advanced molecular biology strategies, have unacceptable side effects due to the drug interacting with healthy tissues that are not the target of the drug. Side effects limit our ability to design optimal medications for

many diseases such as cancer, neurodegenerative diseases, and infectious diseases. Drug delivery systems control the rate at which a drug is released and the location in the body where it is released. Some systems can control both.

The gene and drug delivery systems (GDD) consider grant applications focused on the development and delivery of drugs, genes, and gene products that alter gene function or expression in the living organism. Most studies use tissue culture and/or animal models. Applications are typically driven by bioengineering concepts and may not be hypothesis driven:

- Delivery of nucleic acids, peptide/protein complexes, vaccines, genes, small molecules, antibiotics, theranostics, and other drugs and biomaterials to biological targets
- Delivery vehicles including plasmids, viruses, liposomes, micelles, vesicles, nanoparticles, biomaterials, and cells
- Delivery strategies including electroporation, ultrasound, light, and ballistic methods
- Study of the physiological barriers to delivery (e.g., membrane, tissue, cellular, trafficking, physical)
- Studies of the interactions of delivery vehicles, devices, and/or payloads with the immune system

16.4.3 Biodetection of Pathogens

Advances in DNA sequencing technology have made it possible for scientists all over the world to sequence complete microbial genomes rapidly and efficiently. Access to the DNA sequences of entire microbial genomes offers new opportunities to analyze and understand microorganism at the molecular level. Scientists are able to detect pathogens in biological tissues and study variations in gene expression in response to the pathogenic invasion. These responses help in designing novel approaches for microbial pathogen detection and drug development. Identification of certain microbial pathogens as etiologic agents responsible for chronic diseases is leading to new treatments and prevention strategies for these diseases.

Each species of pathogens carries with it unique DNA or RNA signatures that differentiate it from other organisms. One of the challenges is to develop this DNA signature for each microorganism of interest for rapid and specific detection. Pathogen detection has become an important part of research in many fields like biodefense, animal healthcare, food safety, diagnostics, pathology, clinical research, forensics, and drug discovery. For biodefense, accurate analytical techniques for discovering pathogenic agents are needed. Animal healthcare community uses pathogen detection to develop various diagnostic tests that are rapid, reliable, and highly sensitive for effective control and treatment of diseases of animals. In diagnostics, the technique is employed to detect or identify infectious agents, toxins, parasites, metabolic disorders, and genetic susceptibility/resistance.

16.4.4 Detection of Proteins

The word protein is derived from the Greek *proteios*, meaning "of the first rank." The term was coined in 1838 by the Swedish scientist Jöns Berzelius, to reflect the importance of this group of molecules. SDS polyacrylamide gel electrophoresis (SDS-PAGE) involves the separation of proteins based on their size. By heating the sample under denaturing and reducing conditions, proteins become unfolded and coated with SDS detergent molecules, acquiring a high net negative charge that is proportional to the length of the polypeptide chain. When loaded onto a gel matrix and placed in an electric field, the negatively charged protein molecules migrate toward the positively charged electrode and are separated by a molecular sieving effect. After visualization by a protein-specific staining technique, the size of a protein can be estimated by comparison of its migration distance with that of a standard of known molecular weight.

After protein transfer from an SDS-PAGE gel to a membrane, the remaining protein-free sites on the membrane must be blocked. This prevents the primary or secondary antibody from binding directly to the membrane and giving rise to a high background signal. Several blocking reagents are in common use, including nonfat dried milk, BSA, and casein. After blocking, the primary antibody is added and allowed to bind to the protein. After washing (which removes nonspecifically bound antibody), the secondary antibody is added, to detect where the primary antibody has bound. After another wash step, the location of the secondary antibody (and therefore the primary antibody and the protein of interest) is determined by adding a substrate for the enzyme conjugated to the secondary antibody. Substrates are available that give rise to a colored compound (chromogenic detection), or to the emission of light (chemiluminescent detection), at the reaction site. The use of an antibody that reacts specifically with an epitope commonly introduced into a recombinant protein eliminates the need for a protein-specific antibody and allows the use of one antibody for the detection of all proteins containing this feature. Coupling a reporter enzyme directly to such antibodies eliminates the need for a secondary antibody and delivers significant time savings.

16.4.5 Tissue Engineering

Tissue engineering is the use of a combination of cells, engineering and materials methods, and suitable biochemical and physicochemical factors to improve or replace biological tissues. Tissue engineering involves the use of a scaffold for the formation of new viable tissue for a medical purpose. While it was once categorized as a subfield of biomaterials, having grown in scope and importance, it can be considered as a field in its own.

16.4.6 Tumor Destruction via Heating (Hyperthermia)

Hyperthermia therapy is a type of medical treatment in which body tissue is exposed to slightly higher temperatures to damage and kill cancer cells or to make cancer

cells more sensitive to the effects of radiation and certain anticancer drugs. Techniques that may bring local tissues to quite high temperatures, such as radiofrequency ablation, are not usually what is meant by "hyperthermia." When combined with radiation therapy, it is called thermoradiotherapy. Whole-body hyperthermia has also been found to be helpful for depression (Hanusch et al. 2013). It is also promoted for use in the treatment of chronic Lyme disease.

16.4.7 Separation and Purification of Biological Molecules and Cells

Cell biologists research the intricate relationship between structure and function at the molecular, subcellular, and cellular levels. However, a complex biological system such as a biochemical pathway can only be understood after each one of its components has been analyzed separately. Only if a biomolecule or cellular component is pure and biologically still active can it be characterized and its biological functions elucidated.

Fractionation procedures purify proteins and other cell constituents. In a series of independent steps, the various properties of the protein of interest solubility, charge, size, polarity, and specific binding affinity are utilized to fractionate it or separate it progressively from other substances. Three key analytical and purification methods are chromatography, electrophoresis, and ultracentrifugation. Each one relies on certain physicochemical properties of biomolecules.

16.4.8 MRI Contrast Enhancement

MRI contrast agents are a group of contrast media used to improve the visibility of internal body structures in magnetic resonance imaging (MRI). The most commonly used compounds for contrast enhancement are gadolinium based. Such MRI contrast agents shorten the relaxation times of atoms within body tissues following oral or intravenous administration. In MRI scanners, sections of the body are exposed to a very strong magnetic field causing primarily the hydrogen nuclei ("spins") of water in tissues to be polarized in the direction of the magnetic field. An intense radio-frequency pulse is applied that tips the magnetization generated by the hydrogen nuclei in the direction of the receiver coil where the spin polarization can be detected. Random molecular rotational oscillations matching the resonance frequency of the nuclear spins provide the "relaxation" mechanisms that bring the net magnetization back to its equilibrium position in alignment with the applied magnetic field. The magnitude of the spin polarization detected by the receiver is used to form the MR image but decays with a characteristic time constant known as the T1 relaxation time. Water protons in different tissues have different T1 values, which is one of the main sources of contrast in MR images. A contrast agent usually shortens, but in some instances increases, the value of T1 of nearby water protons, thereby altering the contrast in the image.

16.4.9 Phagokinetic Studies

Tumor cell migration is a key step underlying cancer cell dissemination and metastasis and is controlled by extracellular signaling-mediated dynamic cytoskeletal and cell matrix adhesion remodeling. Using a phagokinetic track (PKT) assay in combination with multi-parametric image analysis and highly motile H1299 adenocarcinoma cells, they have screened 1429 upstream kinase signaling components and downstream adhesion and cytoskeletal regulators that determine tumor cell migratory behavior: speed, directionality, and persistence. Thirty significant genes were validated by live cell imaging random tumor cell migration, which was associated with modulation of focal adhesion dynamics. For eight genes, a significant association with metastasis-free survival in breast cancer patients was observed, SHC1, SRPK1, NEK2, ITGB3BP, and MAP 3K8 being most significant. Also, high SRFS protein kinase 1 (SRPK1) protein expression on breast cancer tissue microarrays was associated with poor disease outcome. SRPK1 expression was highest in basal-like breast cancer cell lines and depletion of SRPK1 inhibited breast cancer cell motility and focal adhesion dynamics. Finally, in an orthotopic mammary tumor metastasis model, stable knockdown of SRPK1 in lung metastatic variant MDA-MB-231 basal-like breast cancer cells reduced lung metastasis formation (Wies van Roosmalen et al 2015). This study provides a comprehensive information resource on the molecular determinants of tumor cell migration in close association with a clinical significant role in breast cancer progression.

As mentioned above, the fact that nanoparticles exist in the same size domain as proteins makes nanomaterials suitable for biotagging or biolabelling. However, size is just one of the many characteristics of nanoparticles that itself is rarely sufficient if one is to use nanoparticles as biological tags. In order to interact with biological target, a biological or molecular coating or layer acting as a bioinorganic interface should be attached to the nanoparticle.

16.5 Summary

A large body of research exists regarding nanoparticles in biology, animal, human, and so on. Following the inventions and the development of industry, however, significant levels of nanoparticle have arisen across large regions in applications. There is heightened concern today that the development of nanotechnology will impact the molecular biology. Research on humans and animals indicates that some nanoparticles are able to enter the body and rapidly migrate to the organs via the circulatory and lymphatic systems. The ability of nanoparticles to enter cells and affect their biochemical function makes them important tools at the molecular level. For example, nanoparticles used to destroy cancer cells or nanoparticles used for soil remediation may have an impact upon entering the food chain via microorganisms, such as bacteria and protozoa. We conclude that the development of nanotechnology is indispensable in the modern molecular biology.

References

Alivisatos P (2004) The use of nanocrystals in biological detection. Nat Biotechnol 22(1):47–52

Allen TM, Cullis PR (2004) Drug delivery systems: entering the mainstream. Science 303(5665):1818–1822

Andrew AM (2000) Nanomedicine, volume 1: basic capabilities, by Robert A. Freitas Jr., Landes Bioscience, Austin, Texas, 1999, xxi+ 509 pp., ISBN 1-57059-645-X Index (Hardback, $89.000), Cambridge University Press. doi: 10.1017/S0263574700232827

Atkinson MA et al (1994) Cellular immunity to a determinant common to glutamate decarboxylase and coxsackie virus in insulin-dependent diabetes. J Clin Investig 94(5):2125

Bachmaier K et al (1999) Chlamydia infections and heart disease linked through antigenic mimicry. Science 283(5406):1335–1339

Balin BJ et al (1998) Identification and localization of Chlamydia pneumoniae in the Alzheimer's brain. Med Microbiol Immunol 187(1):23–42

Bennett-Woods D (2006) Nanotechnology in medicine: implications of converging technologies on humanity. Development 49(4):54–59

Bernardi M et al (2009) Development of metal oxide nanoparticles by soft chemical method. Ceram Int 35(1):463–466

Bhargava V et al (2013) Quantitative transcriptomics using designed primer-based amplification. Sci Rep 3:1740

Bhushan B (2009) Biomimetics: lessons from nature–an overview. Philos Trans R Soc Lond A Math Phys Eng Sci 367(1893):1445–1486

Bhushan B, Jung YC (2011) Natural and biomimetic artificial surfaces for superhydrophobicity, self-cleaning, low adhesion, and drag reduction. Prog Mater Sci 56(1):1–108

Blaser MJ, Chyou P, Nomura A (1995) Age at establishment of Helicobacter pylori infection and gastric carcinoma, gastric ulcer, and duodenal ulcer risk. Cancer Res 55(3):562–565

Bogunia-Kubik K, Sugisaka M (2002) From molecular biology to nanotechnology and nanomedicine. Biosystems 65(2):123–138

Bosch FX et al (1995) Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. J Natl Cancer Inst 87(11):796–802

Bruchez M et al (1998) Semiconductor nanocrystals as fluorescent biological labels. Science 281(5385):2013–2016

Burgos JS (2005) Involvement of the Epstein-Barr virus in the nasopharyngeal carcinoma pathogenesis. Med Oncol 22(2):113–121

Burns MA et al (1996) Microfabricated structures for integrated DNA analysis. Proc Natl Acad Sci 93(11):5556–5561

Cao G (2004) Synthesis, properties and applications. World Scientific, Singapore

Chakrabarti A, Shu L (2010) Biologically inspired design. Artif Intel Eng Des Anal Manuf 24(04):453–454

Chan EY (2005) Advances in sequencing technology. Mutat Res Fundam Mol Mech Mutagen 573(1):13–40

Chan WC, Nie S (1998) Quantum dot bioconjugates for ultrasensitive nonisotopic detection. Science 281(5385):2016–2018

Chen G et al (2016) Nanochemistry and nanomedicine for nanoparticle-based diagnostics and therapy. Chem Rev 116(5):2826–2885

Chesebro B (1998) BSE and prions: uncertainties about the agent. Science 279(5347):42–43

Çiftçioglu N et al (1999) Nanobacteria: an infectious cause for kidney stone formation. Kidney Int 56(5):1893–1898

Colvin VL (2003) The potential environmental impact of engineered nanomaterials. Nat Biotechnol 21(10):1166–1170

Crick F (1970) Central dogma of molecular biology. Nature 227(5258):561–563

Danesh J, Collins R, Peto R (1997) Chronic infections and coronary heart disease: is there a link? Lancet 350(9075):430–436

De La Isla A et al (2003) Nanohybrid scratch resistant coatings for teeth and bone viscoelasticity manifested in tribology. Mater Res Innov 7(2):110–114
deWaard JR et al (2008) Assembling DNA barcodes. In: Environmental genomics. Humana, New York, pp 275–294
Diamanti MV, Pedeferri M (2015) Bioinspired self-cleaning materials. In: Biotechnologies and biomimetics for civil engineering. Springer, Cham, pp 211–234
DiMaio D, Liao JB (2006) Human papillomaviruses and cervical cancer. Adv Virus Res 66:125–159
Disci-Zayed D (2016) Green synthesis of nanoparticles. Christian-Albrechts Universität Kiel, Kiel
Dreher KL (2004) Health and environmental impact of nanotechnology: toxicological assessment of manufactured nanoparticles. Toxicol Sci 77(1):3–5
Edelstein R et al (2000) The BARC biosensor applied to the detection of biological warfare agents. Biosens Bioelectron 14(10):805–813
Elsaesser A, Howard CV (2012) Toxicology of nanoparticles. Adv Drug Deliv Rev 64(2):129–137
Ferrari M (2005) Cancer nanotechnology: opportunities and challenges. Nat Rev Cancer 5(3):161–171
Fohlman J, Friman G (1993) Is juvenile diabetes a viral disease? Ann Med 25(6):569–574
Franzen S, Lommel SA (2009) Targeting cancer with 'smart bombs': equipping plant virus nanoparticles for a 'seek and destroy' mission. Nanomedicine 4(5):575–588
Freitas RA (2002) The future of nanofabrication and molecular scale devices in nanomedicine. In: Studies in health technology and informatics. IOS, Amsterdam, pp 45–60
Freitas RA (2005) Current status of nanomedicine and medical nanorobotics. J Comput Theor Nanosci 2(1):1–25
Freitasjr R (2005) What is nanomedicine? Nanomed nanotechnol Biol Med 1:2
Gillies G et al (2002) A spinal cord surrogate with nanoscale porosity for in vitro simulations of restorative neurosurgical techniques. Nanotechnology 13(5):587
Hammond CJ et al (2010) Immunohistological detection of Chlamydia pneumoniae in the Alzheimer's disease brain. BMC Neurosci 11(1):1
Hanusch K-U et al (2013) Whole-body hyperthermia for the treatment of major depression: associations with thermoregulatory cooling. Am J Psychiatr 170(7):802–804
Hardman R (2006) A toxicologic review of quantum dots: toxicity depends on physicochemical and environmental factors. Environ Health Perspect 114:165–172
He X-S, Shi W-Y (2009) Oral microbiology: past, present and future. Int J Oral Sci 1(2):47
Hebert PD, Cywinska A, Ball SL (2003) Biological identifications through DNA barcodes. Proc R Soc Lond B Biol Sci 270(1512):313–321
Horwitz MS et al (1998) Diabetes induced by Coxsackie virus: initiation by bystander damage and not molecular mimicry. Nat Med 4(7):781–785
Huang X et al (2007) Gold nanoparticles: interesting optical properties and recent applications in cancer diagnostics and therapy. Nanomedicine 2(5):681–693
Iravani S (2011) Green synthesis of metal nanoparticles using plants. Green Chem 13(10):2638–2650
Itzhaki R et al (2004) Infiltration of the brain by pathogens causes Alzheimer's disease. Neurobiol Aging 25(5):619–627
Ivanić K-Z, Tadić Z, Omazić MA (2015) Biomimicry – an overview. Holistic Approach Environ 5(1):19–36
Jain RK, Stylianopoulos T (2010) Delivering nanomedicine to solid tumors. Nat Rev Clin Oncol 7(11):653–664
Janka J, Maldarelli F (2004) Prion diseases: Update on mad cow disease, variant creutzfeldt-jakob disease, and the transmissible spongiform encephalopathies. Curr Infect Dis Rep 6(4):305–315
Jarrett RF (2006) Viruses and lymphoma/leukaemia. J Pathol 208(2):176–186
Jia G et al (2005) Cytotoxicity of carbon nanomaterials: single-wall nanotube, multi-wall nanotube, and fullerene. Environ Sci Technol 39(5):1378–1383
Jiang S et al (2009) NIR-to-visible upconversion nanoparticles for fluorescent labeling and targeted delivery of siRNA. Nanotechnology 20(15):155101
Jung D et al (2013) Chemical biology-based approaches on fluorescent labeling of proteins in live cells. Mol BioSyst 9(5):862–872
Kah JCY et al (2007) Early diagnosis of oral cancer based on the surface plasmon resonance of gold nanoparticles. Int J Nanomedicine 2(4):785

Kahn JS (2006) The widening scope of coronaviruses. Curr Opin Pediatr 18(1):42–47
Kajander EO, Ciftcioglu N (1998) Nanobacteria: an alternative mechanism for pathogenic intra- and extracellular calcification and stone formation. Proc Natl Acad Sci 95(14):8274–8279
Kelsall RW, Hamley IW, Geoghegan M (2005) Nanoscale science and technology. Wiley Online Library, New York
Kneuer C et al (2000) A nonviral DNA delivery system based on surface modified silica-nanoparticles can efficiently transfect cells in vitro. Bioconjug Chem 11(6):926–932
Kol A, Santini M (2004) Infectious agents and atherosclerosis: current perspectives and unsolved issues. Ital Heart J 5(5):350–357
Kostarelos K (2006) The emergence of nanomedicine: a field in the making. Nanomedicine 1(1):1–3
Kramer G, Klingler HC, Steiner GE (2000) Role of bacteria in the development of kidney stones. Curr Opin Urol 10(1):35–38
Kusters JG, van Vliet AH, Kuipers EJ (2006) Pathogenesis of Helicobacter pylori infection. Clin Microbiol Rev 19(3):449–490
Legname G et al (2004) Synthetic mammalian prions. Science 305(5684):673–676
Li M et al (2010) Physiologically based pharmacokinetic modeling of nanoparticles. ACS Nano 4(11):6303–6317
Lipinski C, Hopkins A (2004) Navigating chemical space for biology and medicine. Nature 432(7019):855–861
Lo YD et al (1999) Quantitative analysis of cell-free Epstein-Barr virus DNA in plasma of patients with nasopharyngeal carcinoma. Cancer Res 59(6):1188–1191
Lodish H et al (1995) Molecular cell biology, vol 3. Scientific American, New York
Lynch N et al (2006) PANDAS (paediatric autoimmune neuropsychiatric disorder associated with streptococcal infection). Ir Med J 99(5):155–155
Ma J et al (2003) Biomimetic processing of nanocrystallite bioactive apatite coating on titanium. Nanotechnology 14(6):619
Mah C et al (2000) Microsphere-mediated delivery of recombinant AAV vectors in vitro and in vivo. Mol Ther 1:S239
Majoros IJ et al (2008) Current dendrimer applications in cancer diagnosis and therapy. Curr Top Med Chem 8(14):1165–1179
Marra MA et al (2003) The genome sequence of the SARS-associated coronavirus. Science 300(5624):1399–1404
McIntire LV (2002) World technology panel report on tissue engineering. Ann Biomed Eng 30(10):1216–1220
Mell LK, Davis RL, Owens D (2005) Association between streptococcal infection and obsessive-compulsive disorder, Tourette's syndrome, and tic disorder. Pediatrics 116(1):56–60
Mendonça G et al (2008) Advancing dental implant surface technology–from micron-to nanotopography. Biomaterials 29(28):3822–3835
Mercanzini A et al (2010) Controlled release nanoparticle-embedded coatings reduce the tissue reaction to neuroprostheses. J Control Release 145(3):196–202
Meyer SC (2003) DNA and the origin of life: information, specification, and explanation. Design and Public Education, Darwinism, pp 223–285
Miranda HC et al (2006) Detection of Borna disease virus p24 RNA in peripheral blood cells from Brazilian mood and psychotic disorder patients. J Affect Disord 90(1):43–47
Molday RS, Mackenzie D (1982) Immunospecific ferromagnetic iron-dextran reagents for the labeling and magnetic separation of cells. J Immunol Methods 52(3):353–367
Morange M, Cobb M (2000) A history of molecular biology. Harvard University Press, Cambridge, MA
Muñoz N et al (2003) Epidemiologic classification of human papillomavirus types associated with cervical cancer. N Engl J Med 348(6):518–527
Nakamura K et al (2011) Sequence-specific error profile of Illumina sequencers. Nucleic Acids Res 39(13):gkr344
Nam J-M, Thaxton CS, Mirkin CA (2003) Nanoparticle-based bio-bar codes for the ultrasensitive detection of proteins. Science 301(5641):1884–1886
Nel A et al (2006) Toxic potential of materials at the nanolevel. Science 311(5761):622–627

Nie S (2010) Understanding and overcoming major barriers in cancer nanomedicine. Nanomedicine 5(4):523–528
Nisole S, Stoye JP, Saïb A (2005) TRIM family proteins: retroviral restriction and antiviral defence. Nat Rev Microbiol 3(10):799–808
Nunes SOV et al (2008) RNA from Borna disease virus in patients with schizophrenia, schizoaffective patients, and in their biological relatives. J Clin Lab Anal 22(4):314–320
Ow H et al (2005) Bright and stable core-shell fluorescent silica nanoparticles. Nano Lett 5(1):113–117
Palenik GJ, Jensen WP, Suh I-H (2003) The history of molecular structure determination viewed through the Nobel prizes. J Chem Educ 80(7):753
Pandey A et al (2008) Nanoscience and their biological importance: human health and disease. Dig J Nanomater Biostruct 3(3):141–146
Panessa-Warren B et al (2006) Biological cellular response to carbon nanoparticle toxicity. J Phys Condens Matter 18(33):S2185
Pantarotto D et al (2003) Immunization with peptide-functionalized carbon nanotubes enhances virus-specific neutralizing antibody responses. Chem Biol 10(10):961–966
Parak WJ et al (2002) Cell motility and metastatic potential studies based on quantum dot imaging of phagokinetic tracks. Adv Mater 14(12):882–885
Patel P et al (1995) Association of Helicobacter pylori and Chlamydia pneumoniae infections with coronary heart disease and cardiovascular risk factors. BMJ 311(7007):711–714
Peer D et al (2007) Nanocarriers as an emerging platform for cancer therapy. Nat Nanotechnol 2(12):751–760
Peiris J et al (2003) Coronavirus as a possible cause of severe acute respiratory syndrome. Lancet 361(9366):1319–1325
Pereira P, Monteiro G, Prazeres D (2015) General aspects of biomimetic materials. In: Biotechnologies and biomimetics for civil engineering. Springer, Cham, pp 57–79
Perry AS et al (2013) Insecticides in agriculture and environment: retrospects and prospects. Springer Science & Business Media, Berlin
Prabhu S, Poulose EK (2012) Silver nanoparticles: mechanism of antimicrobial action, synthesis, medical applications, and toxicity effects. Int Nano Lett 2(1):1–10
PROKOP A (2001) Bioartificial organs in the twenty-first century. Ann N Y Acad Sci 944(1):472–490
Quinn S, Gaughran W (2010) Bionics – an inspiration for intelligent manufacturing and engineering. Robot Comput Integr Manuf 26(6):616–621
Ravindran MS et al (2016) Opportunistic intruders: how viruses orchestrate ER functions to infect cells. Nat Rev Microbiol 14(7):407
Riehemann K et al (2009) Nanomedicine – challenge and perspectives. Angew Chem Int Ed 48(5):872–897
Rizzello L et al (2011) Impact of nanoscale topography on genomics and proteomics of adherent bacteria. ACS Nano 5(3):1865–1876
Roco MC (2003) Nanotechnology: convergence with modern biology and medicine. Curr Opin Biotechnol 14(3):337–346
Rota PA et al (2003) Characterization of a novel coronavirus associated with severe acute respiratory syndrome. Science 300(5624):1394–1399
Roy I et al (2005) Optical tracking of organically modified silica nanoparticles as DNA carriers: a nonviral, nanomedicine approach for gene delivery. Proc Natl Acad Sci U S A 102(2):279–284
Sahoo H (2012) Fluorescent labeling techniques in biomolecules: a flashback. RSC Adv 2(18):7017–7029
Salata OV (2004) Applications of nanoparticles in biology and medicine. J Nanobiotechnol 2(1):1
Sarikaya M et al (2003) Molecular biomimetics: nanotechnology through biology. Nat Mater 2(9):577–585
Sawicki MP et al (1993) Human genome project. Am J Surg 165(2):258–264
Schadt EE, Turner S, Kasarskis A (2010) A window into third-generation sequencing. Hum Mol Genet 19(R2):R227–R240
Scott J, Thompson G (2011) The discovery of the structure of DNA. In: Nobel prizes that changed medicine. Imperical College Press, London, p 89

Shendure J, Ji H (2008) Next-generation DNA sequencing. Nat Biotechnol 26(10):1135–1145
Shinkai M et al (1999) Intracellular hyperthermia for cancer using magnetite cationic liposomes. J Magn Magn Mater 194(1):176–184
Slowing II et al (2008) Mesoporous silica nanoparticles as controlled release drug delivery and gene transfection carriers. Adv Drug Deliv Rev 60(11):1278–1288
Smith AM et al (2006) Multicolor quantum dots for molecular diagnostics of cancer. Expert Rev Mol Diagn 6(2):231–244
Swedo SE et al (1998) Pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections: clinical description of the first 50 cases. American Journal of Psychiatry 155(2):264–271
Swihart MT (2003) Vapor-phase synthesis of nanoparticles. Curr Opin Colloid Interface Sci 8(1):127–133
Taton TA, Mirkin CA, Letsinger RL (2000) Scanometric DNA array detection with nanoparticle probes. Science 289(5485):1757–1760
Tokárová V et al (2013) Development of spray-dried chitosan microcarriers for nanoparticle delivery. Powder Technol 235:797–805
Tomb J-F et al (1997) The complete genome sequence of the gastric pathogen Helicobacter pylori. Nature 388(6642):539–547
Variola F et al (2009) Improving biocompatibility of implantable metals by nanoscale modification of surfaces: an overview of strategies, fabrication methods, and challenges. Small 5(9):996–1006
Venter JC et al (2001) The sequence of the human genome. Science 291(5507):1304–1351
Vierra S (2011) Biomimicry: designing to model nature. WBDG. https://www.wbdg.org/resources/biomimicry-designing-model-nature
Vinogradov S, Wei X (2012) Cancer stem cells and drug resistance: the potential of nanomedicine. Nanomedicine 7(4):597–615
Visintin I et al (2008) Diagnostic markers for early detection of ovarian cancer. Clin Cancer Res 14(4):1065–1072
Wagner V et al (2006) The emerging nanomedicine landscape. Nat Biotechnol 24(10):1211–1217
Walther W, Stein U (2000) Viral vectors for gene transfer. Drugs 60(2):249–271
Wang S et al (2002) Antigen/antibody immunocomplex from CdTe nanoparticle bioconjugates. Nano Lett 2(8):817–822
Wang L et al (2012) Nano-hydroxyapatite particles induce apoptosis on MC3T3-E1 cells and tissue cells in SD rats. Nanoscale 4(9):2894–2899
Weil TT, Parton RM, Davis I (2010) Making the message clear: visualizing mRNA localization. Trends Cell Biol 20(7):380–390
Weiler C, Goel AK (2015) From mitochondria to water harvesting: a case study in biologically inspired design. IEEE Potentials 34(2):38–43
Weissleder R et al (1990) Ultrasmall superparamagnetic iron oxide: characterization of a new class of contrast agents for MR imaging. Radiology 175(2):489–493
Wies van Roosmalen et al (2015) Tumor cell migration screen identifies SRPK1 as breast cancer metastasis determinant. J Clin Invest 125(4):1648–1664.
Wolinsky JB, Grinstaff MW (2008) Therapeutic and diagnostic applications of dendrimers for cancer treatment. Adv Drug Deliv Rev 60(9):1037–1055
Yan H et al (2003) DNA-templated self-assembly of protein arrays and highly conductive nanowires. Science 301(5641):1882–1884
Zheng CR et al (2016) Particulate respirators functionalized with silver nanoparticles showed excellent real-time antimicrobial effects against pathogens. Environ Sci Technol 50(13):7144–7151
Zhou H, Lee J (2011) Nanoscale hydroxyapatite particles for bone tissue engineering. Acta Biomater 7(7):2769–2781
Zhu S et al (2002) A novel nonviral nanoparticle gene vector: Poly-L-lysine-silica nanoparticles. Chin Sci Bull 47(8):654–658
Zur Hausen H et al (1970) Epstein-Barr virus in Burkitt's lymphoma and nasopharyngeal carcinoma.[ii] EBV DNA in biopsies of Burkitt tumours and anaplastic carcinomas of the nasopharynx. Nature 228:1056–1058

Nanotechnology and Its Impact on Insects in Agriculture

17

Prashant Singh, Kamlesh Kumari, Vijay K. Vishvakarma, Sangita Aggarwal, Ramesh Chandra, and Anita Yadav

Graphical Abstract



Highlights of the Chapter

- In the present scenario, the researchers paid attention on the nanotechnology and its applications.
- Nanomaterials have been synthesized by various routes with specific size and shape.
- Academicians and researchers explored the nanotechnology in the area of pesticides, herbicides etc.

P. Singh (✉) • V.K. Vishvakarma • S. Aggarwal
ARSD College, University of Delhi, Delhi, India
e-mail: arsdchemistry@yahoo.in, arsdchemistry@gmail.com

K. Kumari
DDU College, Delhi University, Delhi, India

R. Chandra
Department of chemistry, University of Delhi, New Delhi, India

A. Yadav
Rajdhani College, Delhi University, New Delhi, India

D. Kumar, C. Gong (eds.), *Trends in Insect Molecular Biology and Biotechnology*, https://doi.org/10.1007/978-3-319-61343-7_17

- Various potent structures/assemblies or products have been produced with low toxicity and easy synthesis.
- Therefore, there is urgency to explore the nanotechnology in pesticides, herbicides etc.

17.1 Introduction

Nanotechnology has attracted the researchers/scientists/academicians and is considered to be a promising field of multidisciplinary work. This approach has opened a wide way of opportunities to the scientists, industrialists and academicians working in various fields like insecticides, pharmaceuticals, electronics and agriculture. The application of nanotechnology is tremendous and a lot has to be found. Nanotechnology and nanoscience is a multidisciplinary science and deals with design, synthesis and changes in particle structures at a very small scale (nanometre), i.e. 1–100 nm in one or more than one dimension. Nanoparticles (NPs), whether organic/inorganic, have lots and lots of applications in areas like medicine; cosmetics; energy; environment safety; mechanics; various electronic, chemical and space industries; optoelectronics; catalysis; photo-electrochemical applications; and many more.

The mechanism of growth of nanoparticles has been explained in distribution function in terms of their size and their physical-chemical properties. The nanoparticle growth mechanism provides a possibility to control the preparation of nanoparticles and gives the mean diameter, standard deviation, coefficient polydispersity as well as characteristics like magnetic moment. Nanoparticle growth mechanism is a complex process and depends on several conditions like temperature, viscosity and concentration of medium. Conditions applied for the nanoparticle growth vary, depending on the varying methodologies used for the preparation of nanoparticles. Metal nanoparticles (NPs) have gained significant research interest in the past 20 years due to their unique electrical, optical or mechanical properties. They are most appropriate candidate for various applications in various areas of science like optics, catalysis, sensor design, biomedicine, etc. (Herrero et al. 2010; Larguinho and Baptista 2011; Niikura et al. 2013; Prashant et al. 2017; Yao et al. 2012; Zhang et al. 2012). Impressive advances have been achieved in the synthesis of isolated nanostructures. This has opened the possibility for developing a new class of advanced materials with designed properties. Metal NPs have been explored for a number of advanced applications in various areas. Metal NPs have shown optical absorption, and it can be correlated to electronic vibrations pattern on the metal NPs surface. This phenomenon is known as *plasmons*. Literature reported that the morphology of metal NPs does affect the optical properties significantly (Prashant et al. 2012a, b; c; 2014; Pradeep et al. 2011; Prashant 2013; Prashant et al. 2009a, b; Prashant et al. 2011; Prashant et al. 2017).

Plenty of research has been carried out on the size, shape, surface chemistry, etc., of metal NPs. Gold and silver NPs have been observed in the visible ranges. They are chemically noble and environment-friendly. Gold NPs gave an interesting model

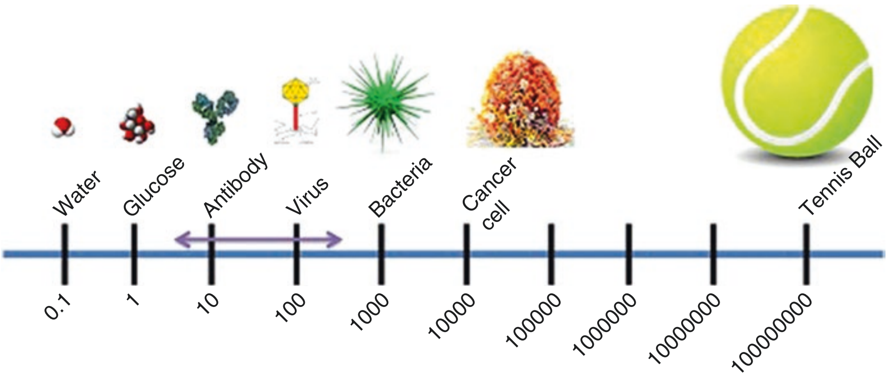


Fig. 17.1 Comparison of various objects in terms of size

system for exploring the world of colloid solutions. Gold NPs have a huge range of applications, like charge storage, sensor and electronic device fabrication. The methodologies for the synthesis of monodisperse metal NPs of particular size and shape are still limited and challenging the scientific community. There is a limited knowledge of mechanisms of NPs formation. The reason for this is the difficulty faced in real-time observation of the formation of NPs. Nucleation and growth processes are often very fast in the synthesis of metal NPs. Therefore, making direct observations is very difficult. Further, literature reported that the melting point of metal nanoparticles depends upon the size. Smaller the size of metal nanoparticles, the more will be the surface area (Kamlesh et al. 2012a, b; Prashant 2013; Prashant et al. 2011) (Fig. 17.1).

There are various routes for the synthesis of metal nanoparticles, and they are physical, chemical, biological and hybrid. Generally in physical methods, two approaches, up-down and down-up, have been studied. Herein, solid-phase synthesis of nanoparticles is taken into consideration. In chemical method, synthesis of NPs has been carried out in the solution phase (Fig. 17.2a). Sometimes, there is a need to use stabiliser to stabilise metal nanoparticle in the solution phase. In biological method, there is no need of stabiliser because the biological extract itself works as stabiliser. Hybrid method uses the combination of two or more than two techniques for the synthesis of the nanoparticle, and this methodology is the most effective one. Various methodologies for synthesising stable metal nanoparticles have been reported. Many research groups specially focus on synthesis methods for creating nanoparticles like physical vapour deposition, chemical vapour deposition, sol-gel method, Radio frequency (RF) plasma method, pulsed laser method, thermolysis and solution combustion method. Various metal nanoparticles have wide range of use in the living and nonliving systems (Kamlesh et al. 2012a, b; 2014, 2017; Pradeep et al. 2011; Prashant 2013; Prashant et al. 2008a, b; 2009a, b, c, 2011, 2017). A general schematic representation of metal nanoparticle can be given in Fig. 17.2b. Herein stabiliser has been used for the synthesis of metal nanoparticle.

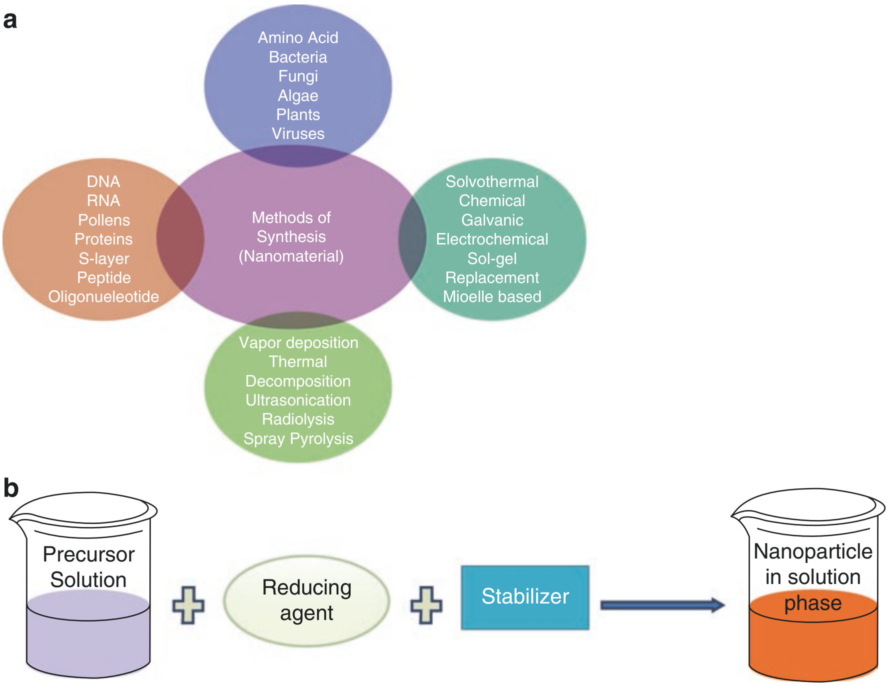


Fig. 17.2 (**a**) Methodologies used for the synthesis of nanoparticles. (**b**) Schematic representation of metal nanoparticle in solution

17.2 Applications of Metal NPs in Various Disciplines

There are several applications of metal NPs as mentioned in Fig. 17.3, but in this section, only few of them are explained (Herrero et al. 2010; Larguinho and Baptista 2011; Niikura et al. 2013; Yao et al. 2012; Zhang et al. 2012).

Role of Metal NPs in Composite Materials A common and popular methodology for the synthesis of metal nanoparticle-polymer composites, this method involves mechanical dispersion of synthesised nanoparticles on a polymeric matrix. Another methodology is reported, and it involves polymerising the matrix around a metal nano-core using chemically compatible ligands. Literature reported the synthesis of silver nanoparticles in PVA matrix through light silver ions getting reduced by the polymeric benzophenone ketyl radicals. A challenge for researchers is the homogenous dispersion of the metal NPs for longer times as well their stability within the matrix. Metal NPs are prone to aggregation also due to their high surface free energy. They get aggregated due to oxidation by air or moisture. This aggregation of metal NPs changes the properties of the metal nanocomposite. Therefore, there is urgency to explore new potential protocols for the preparation of metal nanoparticle.

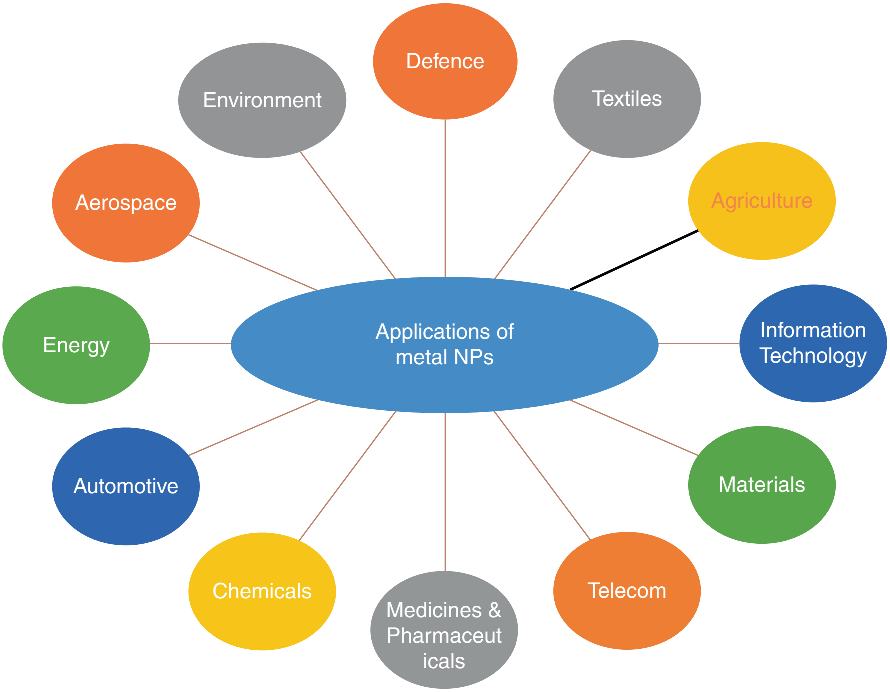


Fig. 17.3 Application of metal NPs in various disciplines (Prashant et al. 2017)

Biomedical Applications of Metal NPs From, first use of metal NPs in ancient times in various medical treatments, paintings, etc and the claims for the use of gold NPs in medical sciences. Literature reported the interesting developments for the use of gold NPs in the delivery of various vaccines into the human body. Au NPs have been used in the finding of deadly poisons, in the testing of vaccines, etc. Au NPs are also used in biomedical analyses. Gold NPs are attractive and efficient material for various testing. This technique gives visual proof of the availability of an analyte in a liquid sample. Au NPs have superiority due to their high stability, sensitivity and reproducibility. Gold NPs have the ability to be layered with particular organic ligands, DNA. It makes easier to engineer nanostructures and to modify properties for different applications. Au NPs have been used to locate the tumours, and when exposed to X-rays, kill the tumour (Prashant et al. 2012a, b, c; 2014; Pradeep et al. 2011; Prashant 2013; Prashant et al. 2009a, b, 2011, 2017).

Biocompatibility One of the important aspects in the study and exploration of nanoscience and nanotechnology is to assess the cytotoxicity levels in the living systems. Several attempts have been made to see the toxicity of metal nanoparticles. Gold nanoparticles have specially been extensively and thoroughly studied for their

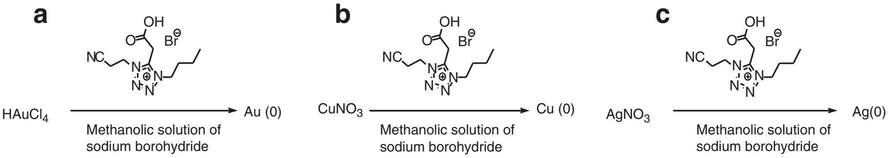
cytotoxicity effects. Au NPs have been explored for various applications in medical science. Researchers have also studied the uptake of gold nanoparticles by mammalian cells by pinocytosis and its compartmentalisation in lysosomal bodies. It was reported that the gold NPs do not show any visible cytotoxicity to the human cell lines until 100 µM concentration. These reports clearly suggest that the gold NPs are biocompatible. Thus, Au NPs can be used in various applications in nanomedicine.

Other Applications of Silver, Gold and Copper Nanoparticles Comparing with organic dyes, the light absorption by Au and Ag NPs is about 5–6 times higher in magnitude, resulting to these metal NPs being used in thermal therapy and optical imaging of tumours. Silver and gold nanostructures were thoroughly investigated as important colorimetric sensors because of their large extinction coefficient. Silver NPs has been used in medicine to decrease the infections in burn treatment, to avoid bacterial colonisation on dental materials and stainless steel materials, to eliminate microorganisms on textile fabrics or to treat water.

17.3 Synthesis and Characterisation of Some Metal Nanoparticles (Ajeet et al. 2008; Chaichi and Alijanpour 2014; Kamlesh et al. 2012a, b, 2014, 2017; Pradeep et al. 2011; Prashant 2013; Prashant et al. 2008a, b, c, 2009a, b, c, 2011, 2017; Scheeren et al. 2006; Xu et al. 2012, 2013; Yang et al. 2014; Zapp et al. 2014)

17.3.1 Synthesis of Au, Cu and Ag NPs in Ionic Liquid (1-Butyl-5-Carboxymethyl-4-(2-Cyano-Ethyl)-4H-Tetrazolium Bromide) (Scheme 17.1a–c)

In a typical methodology, in a 10 mL round bottom flask, 5 mL of the above synthesised ionic liquid and 10 mg of tetrachloroauric acid ($HAuCl_4$) were taken and stirred for 10 min (yellow colour), and the mixture was treated with more methanolic solution of sodium borohydride (20 mg in 10 mL of methanol). A ruby red-coloured solution was obtained from yellow-coloured solution of $HAuCl_4$ indicating the formation of gold in zero oxidation state. Stirring was continued for another 6 hours for the reduction of Au (III) to Au (0). Then the solution obtained was centrifuged for 10 min at 10,000 rpm, and the supernatant was discarded. The centrifuged pellet was washed with ethanol, and the nanoparticles were analysed for characterisation using powder X-ray diffraction (XRD), transmission electron microscopy (TEM), quasi-elastic light scattering (QELS) and UV-visible techniques to determine the shape, size and oxidation state of gold nanoparticles. Similarly, the synthesis of Cu and Ag NPs in ionic liquid (1-butyl-5-carboxymethyl-4-(2-cyano-ethyl)-4H-tetrazolium bromide) (Scheme 17.1b and c, respectively) has been carried out.



Scheme 17.1 Synthesis of Au, Cu and Ag NPs in ionic liquid

17.3.2 Characterisation of Gold, Copper and Silver Nanoparticles (Au NPs)

17.3.2.1 Powder X-Ray Diffraction Analysis of Au, Cu and Ag NPs

Powder X-ray diffraction is also known as non-destructive technique and broadly applied for the characterisation of crystallinity of nanomaterials. This technique is usually practical for data collection under ambient circumstances, but *in situ* diffraction as a purpose of external constraints (temperature, pressure, stress, electric field, atmosphere, etc.) is significant for the interpretation of solid-state conversions and behaviour of material. It explains the nature of the sample (crystallinity, structure imperfections, crystallite size, texture) and the difficulty of the crystal structure (number of atoms in the asymmetric unit cell, unit cell volume). The scaling of powder X-ray diffraction plot is 2θ and intensity counts plotted on x- and y-axes, respectively.

Peak found at 2θ values of 38.1 and 44.2° on *x*-axis is assigned to 111 and 200 crystalline planes and indicates the presence of gold in zero oxidation state (Itoh et al. 2004; Wilcoxon 2009; Xie et al. 2009; Zhang and Cui 2009). On the nanometre scale, metals (most metal NPs are face-centred cubic, or FCC) tend to nucleate and grow into twinned gold nanoparticles and multiply twinned particles with their surfaces bounded. The intensity peak counts indicate the crystalline behaviour of Au NPs. Literature revealed that more the intensity peak counts of metal NPs, more will be its crystallinity. (Fig. 17.4a). As per the pattern of powder X-ray diffraction (Fig. 17.4b), the 2θ values 38.1, 44.2 and 64.8° clearly signify the presence of copper in zero oxidation state in the sample. The conformation of metal nanoparticle was due to the results of JCPDS data. The value of 2θ at 44.2° and 50.2° corresponds to 211 and 320, which tells that Cu is in cubic form. Power X-ray diffraction plot was obtained from 5 to 60 (2θ value), and high intensity peak counts showed the crystalline behaviour of silver nanoparticle (Fig. 17.4c). Powder X-ray diffraction confirms the present of Ag in zero oxidation state due to 2θ value at 37.2 and 45.1. Further, the intensity peak counts indicate the crystalline nature of Ag NPs.

17.3.2.2 Transmission Electron Microscopy (TEM) Analysis of Au, Cu and Ag NPs

Transmission electron microscopy (TEM) is a vital characterisation technique for directly imaging the nanomaterials to obtain quantitative measures of nanoparticle and/or grain size, size distribution and morphology. In other words, it

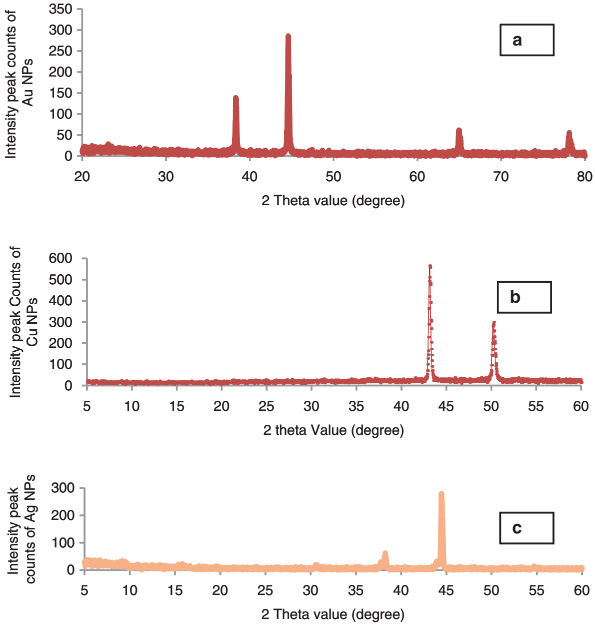


Fig. 17.4 Powder X-ray diffraction of (**a**) Au, (**b**) Cu and (**c**) Ag NPs

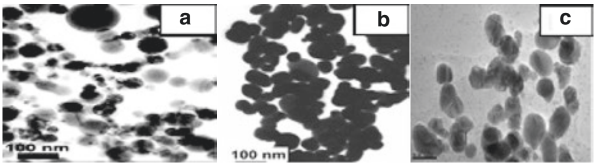


Fig. 17.5 TEM pictures of (**a**) Au, (**b**) Cu and (**c**) Ag NPs

determines the shape and size of the Au nanoparticles. On the analysis of TEM data, it was observed that the range of size of gold NPs is 20–30 nm and the particles are spherical in nature as in Fig. 17.5a. On the analysis of TEM picture of Cu NPs, shape of the NPs appeared spherical and the bar present in the picture corresponds to 100 nm and corresponds to approximately two nanoparticles; therefore, the average particle size of the particles is 50–60 nm (Fig. 17.5b). Transmission electron micrographs of the sample clearly indicate that the size of Ag NPs is approximately 50 nm with spherical shape (Fig. 17.5c).

17.3.2.3 Quasi-elastic Light Scattering (QELS) Analysis of Au, Cu and Ag NPs

This technique is used to evaluate the average size of NPs and their dispersive behaviour in a particular solvent as this technique is applicable for solutions. QELS data indicate that the nanoparticles are monodispersive and the average size of the Au NPs is 30.50 nm as in Fig. 17.6a, which was in correlation with TEM data. On the analysis of QELS pictures (Fig. 17.6b) and data, it was found that the Cu NPs are

monodispersive in nature and the average size of the NPs was found out to be 55.47 nm, which is in correlation with TEM results as explained above. QELS pictures (Fig. 17.6c) gave clear indication that Ag NPs are monodispersive and the average size of the particles is 57.31 nm, which is again in correlation with TEM results.

17.3.2.4 UV-Visible Spectral Analysis of Au, Cu and Ag NPs

This technique is used to determine the size of NPs and explain the plasmon behaviour. It means that it is used to study the effect of size on the colour of NPs. It was found that as the size of Au NPs decreases, λ_{max} increases or shifts towards higher wavelength. The λ_{max} at 545 nm indicates the size of Au NPs is 25–30 nm (Fig. 17.7a). Reduction of Cu^{2+} ions in ionic liquid using ethanolic solution of $NaBH_4$, a brownish black solution of Cu NPs, is obtained and gave a characteristic peak 573 nm and indicates the size of NPs in the range of 50–60 nm (Fig. 17.7b). As the size of NPs decreases, λ_{max} also increases or shifts to higher wavelength. Reduction of Ag^{+} ions in ionic liquid using ethanolic solution of $NaBH_4$, a yellowish brownish solution of

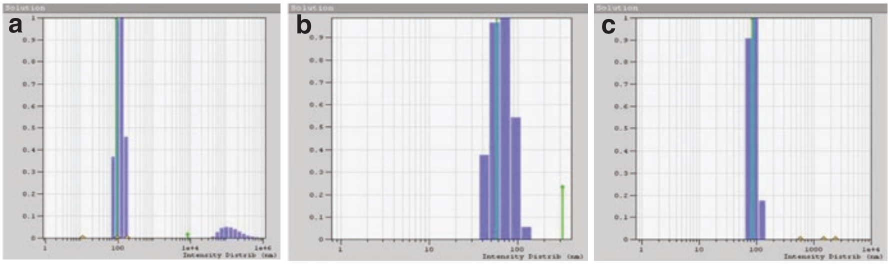


Fig. 17.6 QELS pictures of (**a**) Au, (**b**) Cu and (**c**) Ag NPs

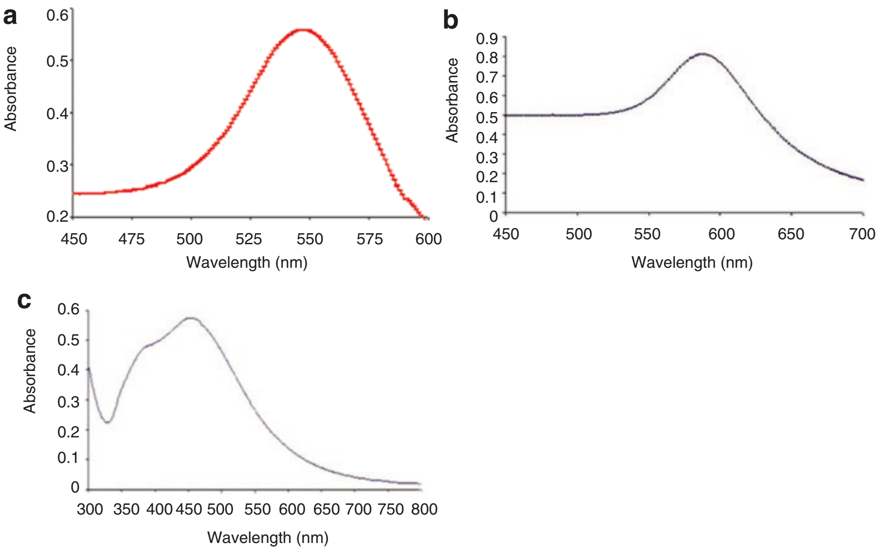


Fig. 17.7 UV-visible spectra of (**a**) Au, (**b**) Cu and (**c**) Ag NPs

Ag NPs, is obtained and gave a characteristic peak of 460 nm and indicates the size of NPs is in the range of 50–60 nm (Fig. 17.7c). As the size of NPs decreases, λ_{max} also increases or shifts to higher wavelength.

17.4 What Is Meant by Pest and the Way to Control?

Insects are counted as one of the major animal populations with a long successful evaluative history. They can even be found in all possible environments throughout the world. Their success can be attributed to several important evolutionary aspects like wings, malleable exoskeleton, high reproductive potential, habit diversification, desiccation-resistant eggs and metamorphosis. On the other hand, many insects are known as vectors of many diseases. Insects damages crop plantations or wood structures, and serious health and economic issues were caused by insects. In order to combat the various losses that are caused by insects on agriculture, several chemicals have been used to kill them or inhibit their reproduction and feeding habits (Buonasera et al. 2009; Ebrahimi et al. 2011; Ghormade et al. 2011; Ginet et al. 2011; Ginjupalli and Baldwin 2013; Grillo et al. 2016; Hake et al. 2007; Jokar et al. 2016; Kah and Hofmann 2014). Nanopesticides encompass a great variety of products and cannot be considered as a single category. Nanopesticides can consist of organic ingredients and inorganic ingredients. The aims of nanoformulations consist of (1) increasing the apparent solubility of poorly soluble active ingredient and (2) releasing the active ingredient in a slow/targeted manner as well protecting the active ingredient against premature degradation. Literature also defines nanopesticides as any formulation that intentionally includes elements in the nanoscale size range and has novel properties. The advances in science and technology in the last two decades were made in several areas of insecticide usage. It includes either development of more effective and non-persistent pesticides and new ways of application, like controlled release formulation. One of the most promising uses of nanotechnology is to promote a more efficient assembly of the active compound in a matrix in order to protect core materials from adverse reactions due to factors like air or light. An outcry is exhibited against the use of pesticides due to their hazardous effects on human as well as environment. Insecticides are proven to be a cheap approach to managing disease-carrying, crop-destroying, and residential pest insects. Chemical insecticides have a long evolutionary history spanning sulphur as a fumigant against vermin to current neonicotinoids. Neonicotinoids are introduced as a new class of insecticides in 1991. They control the agonist effects of the nicotinic ACh receptor in insects. These chemicals also act reversibly on the $\alpha4\beta2$ subtype of mammalian nicotinic ACh receptors and display some chronic toxicity in mammals. Hence, their use to protect a variety of agricultural crops has increased during the last 20 years (Hubert et al. 2007; Hummel et al. 2013; Kah et al. 2014, 2016; Khandelwal et al. 2016; Khodadoust et al. 2013; Kookana et al. 2016; Kumar et al. 2016). The development of novel plant-protection products has received greater attention than other applications, such as those related to nanosensors or fertilisers (Hubert et al. 2007; Hummel et al. 2013; Kah et al. 2014, 2016; Khandelwal

Table 17.1 Environmental fate of various nanoformulations reported in literature published between October 2011 and October 2013

Type and AI	Fate of the nanoformulation compared to that of a commercial formulation or the pure AI	References
Polymer based on polyethylene glycol imidacloprid	Slower release in water than commercial formulation	Adak et al. (2012)
Thiamethoxam	β-cyfluthrin complex	Kaushik et al. (2013)
Carbofuran	More rapid release with increasing PEG molecular weight	Loha et al. (2011)
Thiamethoxam	Slower release in soils compared to commercial formulation	Sarkar et al. 2012
Emamectin	Enhanced photostability compared to commercial formulation	Sarkar et al. (2012)
Lansiumamide B	Enhanced photostability compared to pure AI	Qing et al. (2013)
Paraquat	Weaker sorption than pure AI	Yin et al. (2012)
Pheromones	Reduced volatilisation compared to pure AI	Silva et al. (2011)
Deltamethrin	Decreased direct and indirect photodegradation compared to pure AI	Bhagat et al. (2013)

AI active ingredient

et al. 2016; Khodadoust et al. 2013; Kookana et al. 2016; Kumar et al. 2016; Mirabelli et al. 2016; Mishra et al. 2016) (Table 17.1).

17.5 What Do You Meant by Nanopesticides?

It is important to distinguish the different ways in which the term "nanopesticide" is used within regulatory, scientific, public and commercial frameworks. They have been applied in different contexts and typically vary with regard to, for example, particle size and activity. The need for a regulatory definition of nanopesticides is becoming increasingly recognised. The recurrent question is whether nanopesticides are already on the market or not. It cannot be answered until a clear definition has been agreed. The US EPA is often considered to be the first regulatory authority to have recognised the issue of nanopesticides. The FIFRA Scientific Advisory Panel was consulted by the EPA concerning the evaluation of metal NP-based pesticide products. In 2011, the EPA granted a conditional registration for the first nanosilver pesticide. It is important to note, however, that the product registered is an antimicrobial agent designed for use in clothing, and not intended for application to crops (Hubert et al. 2007; Hummel et al. 2013; Kah et al. 2014, 2016; Khandelwal et al. 2016; Khodadoust et al. 2013; Kookana et al. 2016; Kumar et al. 2016; Mirabelli et al. 2016; Mishra et al. 2016). Literature reported that the nanopesticides to be used as all plant-protection products should (1) intentionally include entities in the nm size range, (2) are designated with a "nano" prefix and (3) are claimed to exhibit novel properties associated with the small size of their components. An overview of the potential applications of nanotechnology within the pesticide

formulation sector as well as definitions and illustrations for the different nanopesticide types can be found (Mirabelli et al. 2016; Mishra et al. 2016; Narayanan et al. 2017; Nuruzzaman et al. 2016; Periasamy et al. 2009; Petosa et al. 2016; Wang et al. 2015, 2016; Yu et al. 2007; Zheng et al. 2016) (Table 17.2).

Table 17.2 Efficacy and toxicity of nanoformulation reported in literature published between October 2011 and October 2013

Type of AI	Efficacy of the nanoformulation compared to that of a commercial formulation or the pure AI	References
Neem oil	IC50 decreased with droplet size	Anjali et al. (2012)
Permethrin	Greater efficacy than pure AI against larvae (LC50) (24 h), 0.006 and 0.020 mg/L, respectively	Kumar et al. (2013)
Glyphosate	Similar or slightly greater efficacy than commercial (roundup)	Jiang et al. (2012)
Beta-cyfluthrin	Greater efficacy than commercial formulation when evaluated over a long period	Loha et al. (2012)
Acephate	Greater efficacy than commercial formulation (biochemical assays, in vitro and field trials)	Choudhury et al. (2012)
Emamectin	Similar and slightly higher insecticidal activity than commercial formulation	Qian et al. (2011)
Lansiumamide B	Higher nematicidal activity than pure AI	Yin et al. (2012)
Neem oil	Cytotoxicity (human lymphocyte): alginate < starch < polyethylene glycol formulation	Jerobin et al. (2012)
Ametryn	Lower toxicity than pure AI	Grillo et al. (2012)
Atrazine and simazine nanogel	Efficacy in open orchard demonstrated during adverse season	Abreu et al. (2012)
Essential oil	Greater efficacy than free oil	Silva et al. (2011)
Copper	Synergistic effect between chitosan nanogels and copper	Brunel et al. (2013)
Thiamethoxam	Efficient at 50% of the recommended dosage	Xiang et al. (2013)
Pyrifluquinazon	Delayed efficacy compared to pure AI	Kang et al. (2012)
Etofenprox	Prolonged effect compared to commercial formulation	Hwang et al. (2011)
Chlorfenapyr	Insecticidal activity of silica nanoparticles formulation was twice as high as that of microparticles	Song et al. (2012)
Naphthylacetic acid	Better growth protection than pure AI after 7 days at the highest concentration tested	Mingming et al. (2013)
Validamycin	Most Prolonged activity compared to pure AI	Qing et al. (2013)
Inorganic silica as AI silica	Efficacy at similar rates than commercial diatomaceous earth for stored gains	Debnath et al. (2011)
TiO_2	Better or on per efficacy compared to standard treatment	Debnath et al. (2012) and Paret et al. (2013a)
Silver	Fungicidal activity against 18 plant pathogens	Paret et al. (2013b)
Copper	Greater efficacy than Cu oxychloride	Kim et al. (2012)
Aluminium	Similar or greater insecticidal activity than most effective commercial available diatomaceous earth formulation	Mondal and Mani (2012) and Stadler et al. (2010)

LC 50, EC 50: Concentration required to observed 50% mortality or effect, respectively

The development of novel plant-protection products has received greater attention than other applications, such as those related to nanosensors or fertilisers. This perspective focuses on the applications of nanotechnology for plant protection and nutrition, in the form of nanopesticides or nanofertilisers. The use of agrochemicals is crucial in modern agriculture, but the development of nanopesticides and nanofertilisers has received less or at least delayed attention relative to other sectors of the food chain, such as food processing or packaging. Due to their direct and intentional application in the environment, nanoagrochemicals may be regarded as critical in terms of possible environmental impact, because they represent the only intentional diffuse source of engineered nanoparticles in the environment. Inventories presented to date and based on patent analysis and scientific literature indicate that the terms can designate a very wide range of products regarding size, nature, level of development and even relevance for agricultural practices. In the scientific literature, the prefix “nano” has been associated until now with the notion of novelty and implicitly suggests superior properties relative to non-nano counterparts. Hence, many formulations were named “nano” with the main objective of increasing attention and possibly facilitating publication. When the information makes its way to non-specialist readership, there is a risk of confusion about what a nanopesticide or a nanofertiliser is and how it relates, for instance, to the definitions that have been proposed for regulatory purposes (Mirabelli et al. 2016; Mishra et al. 2016; Narayanan et al. 2017; Nuruzzaman et al. 2016; Periasamy et al. 2009; Petosa et al. 2016; Wang et al. 2015, 2016; Yu et al. 2007; Zheng et al. 2016).

These include management of insect pests through the formulations of nanomaterial-based insecticides. Traditional strategies like integrated pest management used in agriculture are insufficient, and application of chemical pesticides has adverse effects on animals and human beings apart from the decline in soil fertility. Therefore, nanotechnology would provide green and efficient alternatives for the management of insect pests in agriculture without harming the nature. This art is focused on traditional strategies used for the management of insect pests and potential of nanomaterials in insect pest control as modern approaches of nanotechnology (Belz et al. 2017; Ellison et al. 2017; Hu et al. 2016; Kalayou et al. 2016).

17.6 Application of NPs As Insecticides (Tables 17.1 and 17.2)

We focus on the role of various metallic nanoparticles as potential antimicrobials and the possible mechanism of their inhibitory action (Hu et al. 2016; Hubert et al. 2007; Hummel et al. 2013; Kah and Hofmann 2014; Kah et al. 2016; Kalayou et al. 2016; Khandelwal et al. 2016; Khodadoust et al. 2013; Kookana et al. 2016; Kumar et al. 2016; Mirabelli et al. 2016; Mishra et al. 2016; Nuruzzaman et al. 2016; Periasamy et al. 2009).

17.6.1 Silica

Silicon has long been known to increase the plant tolerance of various abiotic and biotic stresses. Silica nanoparticles have therefore naturally been suggested as potential candidates for increasing the control over a range of agricultural pests. It was observed that there is higher insect mortality from treatment with silica nanoparticles (15–30 nm) than with bulk silica (100–400 nm). The similar efficacy of silica nanoparticles with different coatings indicated a mechanical mode of action that could be enhanced for smaller particles. A second study, however, indicated that silica nanoparticles coated with 3-mercaptopropyltriethoxysilane were more efficient than those coated with hexamethyldisilazane. In one case, the effect was not related to size since the former nanoparticles (29–37 nm) were larger than the latter (15–20 nm). The application rates were generally comparable with those recommended for commercially available diatomaceous formulations (0.5–2 g/kg), and hence the additional costs involved in engineering nanoparticles may not be justified by the slight increase in efficacy (Belz et al. 2017; Ellison et al. 2017; Laranjeira et al. 2017; Xiang et al. 2017; Yao et al. 2017; Zheng et al. 2017).

17.6.2 Titanium Dioxide (TiO_2)

The antimicrobial activity of titanium dioxide is well recognised, and several studies have suggested that applying titanium dioxide to crops can suppress bacterial and fungal pathogens. The antibacterial potential of photocatalytic nanoscale titanium dioxide has recently been tested. Nanoscale titanium dioxide has been used either alone or doped with silver or zinc, against the causal agent for bacterial spot disease in tomatoes and roses. Greenhouse and field trials showed that using titanium dioxide/zinc could result in significantly reduced bacterial spot severity compared to using untreated controls. The overall efficacy was better than the standard treatments for management of the diseases. The main advantage of the titanium dioxide/zinc formulation presented is its potential to lower ecological and toxicological risks, compared to currently use copper-based treatments (Abreu et al. 2012; Anjali et al. 2012; Bhagat et al. 2013; Brunel et al. 2013; Choudhury et al. 2012; Grillo et al. 2012; Hwang et al. 2011; Jerobin et al. 2012; Jiang et al. 2012; Kang et al. 2012; Kumar et al. 2013; Loha et al. 2012; Qian et al. 2011; Silva et al. 2011; Xiang et al. 2013; Yin et al. 2012).

17.6.3 Silver

Silver is known for a long time for its antimicrobial properties, and several in vitro studies have demonstrated that nanosilver can significantly inhibit the growth of plant pathogens in a dose-dependent manner. Recently people demonstrated the *in vitro* activity of nanosilver against 18 plant pathogens. Where possible uses are

coatings for fruit bags or as treatments for cut flowers are conceivable, the application of nanosilver to crops that are likely to enter the food chain is more questionable. Possible benefits of nanosilver over synthetic fungicides have been suggested in view of the cost of nanosilver, the uncertainties associated with its toxicity. According to the latest regulatory developments and the public perceptions, it is unlikely that formulations of nanosilver for open field application will be developed. This conclusion is corroborated by the lack of recent publications on the subject (Debnath et al. 2011, 2012; Hwang et al. 2011; Kim et al. 2012; Mingming et al. 2013; Mondal and Mani 2012; Paret et al. 2013a; Song et al. 2012; Stadler et al. 2010, 2012).

17.6.4 Copper

It is reported that a nanoformulation of copper could suppress the growth of bacterial blight on pomegranate at concentrations of 0.2 mg/L, four orders of magnitude lower than that usually recommended for copper oxychloride (2500–3000 mg/L). This result can be compared to the only other nanocopper formulation which exhibited an 8% increase in efficiency compared to a formulation of copper hydroxides salts currently in use. Researchers carried out *in vitro* and no details of the formulation were provided. This comparison therefore highlights the importance of testing nanoformulations under a range of conditions that are as realistic as possible and of providing the characteristics of the nanoformulations so that further comparisons can be made (Abreu et al. 2012; Anjali et al. 2012; Bhagat et al. 2013; Brunel et al. 2013; Choudhury et al. 2012; Grillo et al. 2012; Hwang et al. 2011; Jerobin et al. 2012; Jiang et al. 2012; Kang et al. 2012; Kumar et al. 2013; Loha et al. 2012; Mingming et al. 2013; Qian et al. 2011; Silva et al. 2011; Song et al. 2012; Xiang et al. 2013; Yin et al. 2012).

17.6.5 Aluminium Oxide (Al_2O_3)

The application of nanostructured alumina dust has been proposed to protect stored grains. Preliminary experiments showed insecticidal activity of alumina NPs at rates comparable to those recommended for commercially available insecticidal dusts. Further, the activity of nanoalumina has been compared to that of the most effective diatomaceous earth formulation on the market. Results obtained for two insect species and at three humidity levels showed that nanoalumina was equally effective as, or more effective than the commercial formulation. Nanoalumina may thus be a good alternative to products based on diatomaceous earth. However, the mode of action of nanoalumina has yet to be elucidated, and further research will be required to optimise the product in terms of the mineral composition of the dust and the type of formulation, and in order to ensure efficacy for a range of insect species and under a range of environmental conditions (Stadler et al. 2010, 2012).

17.6.6 Zinc Oxide (ZnO)

Zinc oxide nanoparticles are very much important due to their utilisation in gas sensors, biosensors, cosmetics, drug delivery systems and so forth. ZnO NPs also have remarkable optical, physical and antimicrobial properties and therefore have great potential to enhance agriculture. The zinc oxide nanoparticles are integrated in pest management programmes as alternative to chemical insecticides where they are considered safe for humans compared with synthetic insecticide. ZnO NPs can be synthesised by several chemical methods such as precipitation method, vapour transport method and hydrothermal process. The biogenic synthesis of ZnO NPs by using different plant extracts is also common nowadays.

This green synthesis is quite safe and eco-friendly compared to chemical synthesis. Several novel inventions of different nanoparticles and nanomaterials are capable to diminish the environmental problems. Nanopesticides develop and explore the possibility of nanotechnology, which accentuate the concept of particle size reduction and its properties. Nanoencapsulation is another part of nanotechnology in which the pesticide is coated by a matrix and the size of the pesticide reduces up to the nano size. Therefore, the nanoencapsulation helps to minimise the doses to get maximum effect on the target organisms. In recent years, it is emphasised on the application of nanotechnology in insect pest management. Technologies like encapsulation and controlled release system (CRS) have, therefore, modernised the application of biocides. Nanotechnology-based insecticides are devised by a number of companies. These formulations embrace nanoparticles of size 120–250 nm size range being more efficiently water soluble as compared to existing pesticides. In India, the relevance of nanotechnology in pest management has just started in the last two decades.

Mosquito-borne diseases are one of the world's most health perilous problems. Numerous mosquito species belonging to genera *Aedes*, *Anopheles*, and *Culex* are the common vectors which cause various diseases like dengue, yellow fever, malaria, filariasis, Japanese encephalitis, etc.; lot of efforts have been made to control the mosquitoes. Initially chemical insecticides like dichlorodiphenyltrichloroethane (DDT), benzene hexachloride (BHC), malathion, etc., have been used to control the mosquito inhabitants, but these chemicals cause ill effects on the environment and non-target organisms and are also non-biodegradable in nature. Therefore, environment friendly NPs were used to control the mosquito population instead of using chemical NPs. Since antiquity plant products have been exposed to display not only their pharmacological benefits but also for other biological properties including fungicidal, microbial, insecticidal and pesticidal activities. Therefore, the botanical pesticides were initiated for further research as they are effective, eco-friendly, easily biodegradable and non-toxic to non-target organisms. The encapsulated plant-based nanopesticides have the following advantages: they can be easily taken by target organisms and are more active in action as compared to synthetic nanopesticides. Plant-based nanopesticides are eco-friendly since they are biodegradable. Pests and vectors were unsuccessful to develop resistance against

plant-based nanopesticides. Nanoencapsulated pesticides are with control release, have long shelf life and cause less pollution. As compared to synthetic-based nanopesticides, the encapsulated plant-based nanopesticides attract extra attention and sincere efforts should be made for the development of plant-based nanopesticides.

17.7 Objection of Regulators on the Usage of Nanoagrochemicals

Literature revealed that the majority of publications originated from mainly Asia, from China and India, followed by the United States. About 55% of the nanopesticides investigated were insecticides, then fungicides (30%) and followed by herbicides (15%). In general, the hypothesis is as follows: the smaller the size, the higher is the reactivity. Therefore, more reactive materials cannot be agrochemicalised. Most of the nanopesticides come under "nano", but literature reported that 100 nm size is the boundary that has been recommended for regulatory purposes. There are several considerable issues relating to the definition of nanoparticles and how the criteria proposed could apply to nanopesticides. Most importantly, a definition based on size alone would exclude many recent so-called nanoformulations and, on the other hand, include products that have been on the market for decades without posing particular. The European Union (EU) initiative of a repository for nanomaterials (EC 2014), therefore, comes with the risk to further confuse consumers by including ingredients that have been used for decades without previously being classified as "nano". In this context, it may be more useful to speak about nano-enabled or formulation technology, rather than focusing only on the nanoparticles and how they should be defined (Balaji et al. 2015, 2017; Buonasera et al. 2009; Diaz-Blancas et al. 2016; Ghormade et al. 2011; Grillo et al. 2016; Hu et al. 2016; Hubert et al. 2007; Hummel et al. 2013; Kah 2015; and Hofmann 2014; Kah et al. 2016; Kalayou et al. 2016; Khandelwal et al. 2016; Khodadoust et al. 2013; Kookana et al. 2016; Kumar et al. 2016; Mirabelli et al. 2016; Mishra et al. 2016; Nuruzzaman et al. 2016; Periasamy et al. 2009) (Fig. 17.8).

17.8 Availability of Nanoagrochemicals in Decade

Literature has reported many nanoagrochemicals, and they do not fit due to certain limitations. Most of the nanoagrochemicals have low agronomic relevance, and the rest has association with unacceptable risks without any advantages. Engineered nanoparticles have attained lots and lots of interest in many areas which have very low potential for large-scale agricultural applications. Nanoagrochemicals having organic-based delivery systems have been used for food or pharmaceutical applications. However, they are economically incompetitive in comparison to agrochemicals. Many studies have been reported about the potency of products whether they

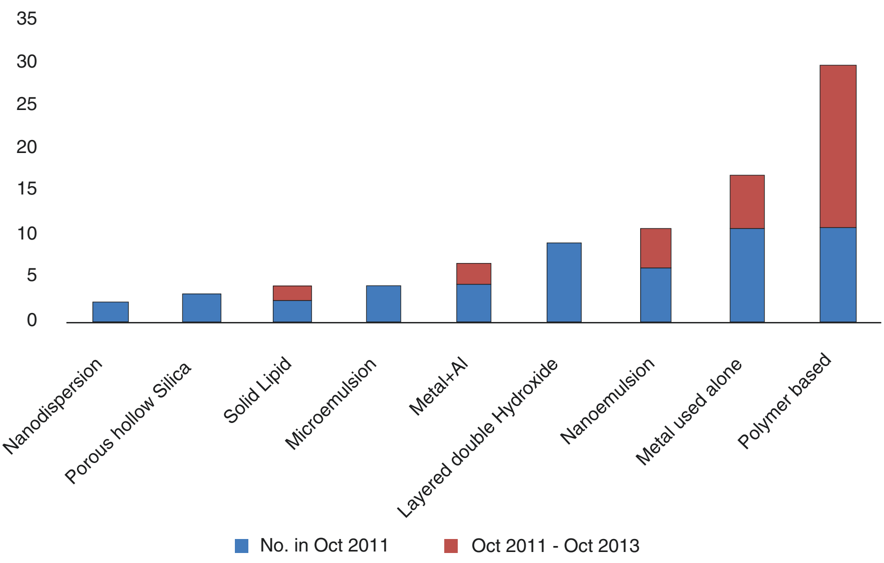


Fig. 17.8 Chart diagram for the different agrochemicals in different span of time

are capable to compete with the present formulations (costs and performance). As a whole, nanoagrochemicals will soon emerge consisting of "nano" formulations of ingredients (Balaji et al. 2015, 2017; Buonasera et al. 2009; Diaz-Blancas et al. 2016; Ghormade et al. 2011; Grillo et al. 2016; Hu et al. 2016; Hubert et al. 2007; Hummel et al. 2013; Kah and Hofmann 2014; Kookana et al. 2016; Kumar et al. 2016; Mirabelli et al. 2016; Mishra et al. 2016; Nuruzzaman et al. 2016; Periasamy et al. 2009).

Development of new formulations has remain an interesting research area. Literature reported that there is a need to formulate agrochemicals of specific applications. With increasing pressure of regulatory agencies, the researchers have focused on the optimisation application and delivery to authorised active ingredients. Researchers are working on the formulation to find out new solutions for targeting agrochemical activity. Keeping the view to maintain colloidal stability as well to avoid the phase separation during storage, many formulations contain structures having size less than 100 nm. Therefore, researchers have access to the advanced instruments or technology that permits clear picture. This facilitates the synthesis and modifications for a separate purpose. Nano-based products have the capability to support a better management of agricultural inputs. Hence, the marketing of formulation of the active molecules on the market should not be based on its size, but also depends on evaluation of new risks and benefits involved therein (Balaji et al. 2015; Buonasera et al. 2009; Diaz-Blancas et al. 2016; Ghormade et al. 2011; Grillo et al. 2016; Hu et al. 2016; Kookana et al. 2016; Kumar et al. 2016; Mirabelli et al. 2016; Mishra et al. 2016).

17.9 Polymer-Based Nanopesticides

Recent publications on polymer-based nanopesticides have been categorised into two ways. The first category involves studies in the fate and efficacy of nanopesticides, wherein the synthetic methodology has been published already. The research is the second category to find the probable new applications of polymer nanoscience for the protection of plant products (Balaji et al. 2015; Buonasera et al. 2009; Diaz-Blancas et al. 2016; Efremenko et al. 2017; Ghormade et al. 2011; Ginet et al. 2011; Grillo et al. 2016; Hu et al. 2016; Hubert et al. 2007; Jokar et al. 2016; Kah and Hofmann 2014; Kah et al. 2014, 2016; Kamlesh et al. 2012a; Kookana et al. 2016; Kumar et al. 2016; Mirabelli et al. 2016; Mishra et al. 2016) (Table 17.3).

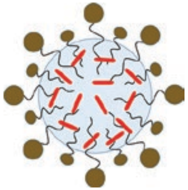
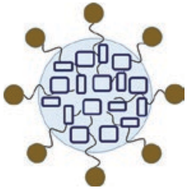
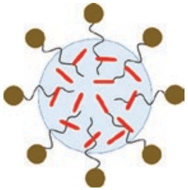
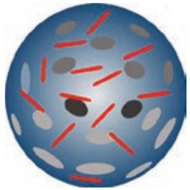
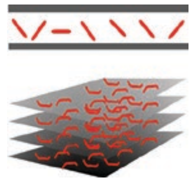
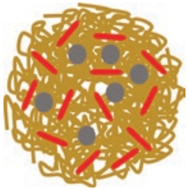
17.10 Release and Efficacy

Literature reported that the researchers are actively engaged in developing and testing a series of insecticide formulations based on amphiphilic copolymers. They found that the active ingredient (AIs) in water were less active than from commercial formulations including imidacloprid, thiamethoxam, carbofuran, thiram and β-cyfluthrin. It has been observed that the release rates increases on increasing molecular weight of Polyethylene glycol (PEG). It was found that the release of β-cyfluthrin from the nanoformulation has occurred in 1–20 days while its release from the available commercial formulation happened in 4–5 days. The high efficacy may be explained by a slow release of AIs as well as protection of the active ingredients, but in case of nanoemulsions, the reasons for showing the less toxicity to non-target organisms are still not clear (Buonasera et al. 2009; Diaz-Blancas et al. 2016; Efremenko et al. 2017; Grillo et al. 2016; Kah and Hofmann 2014; Kookana et al. 2016; Momeni and Nabipour 2015).

17.11 Hybrid Inorganic Nanoparticles with Organic AIs

To date the formulations reported in this category involve the following: (1) using mesoporous silica as a carrier for decreasing the rate of slow release or (2) incorporating TiO_2 onto a polymer matrix to accelerate the photodegradation of the organic AIs. Few new formulations have been reported and have used nanoparticle of silica or calcium carbonate as carriers. A slow release of an organic AI has been shown. These nanoformulations have reported to increase the activity of the AIs, but no generalisations are yet possible due to the very different natures of the AIs investigated (Abreu et al. 2012; Anjali et al. 2012; Bhagat et al. 2013; Brunel et al. 2013; Choudhury et al. 2012; Debnath et al. 2011, Debnath et al. 2012; Grillo et al. 2012; Hwang et al. 2011; Jerobin et al. 2012; Jiang et al. 2012; Kang et al. 2012; Kim et al. 2012; Kumar et al. 2013; Loha et al. 2012; Mingming et al. 2013; Mondal and Mani 2012; Paret et al. 2013a; Qian et al. 2011; Qing et al. 2013; Sarkar et al. 2012;

Table 17.3 Micro-, nano- emulsion, entrapment

Increasing the solubility of poorly water-soluble AI			
Micro-emulsion (6–50 nm)	Nanoemulsion (20–200 nm)	Nano-dispersion (50–200 nm)	
			
Slow/targeted release and protection against premature degradation			
Soft matrix		Hard matrix	
Polymer based (10–30 nm)	Solid lipid (200 nm–100 μm)	Porous hollow silica (100–200 nm)	LDH and clays (μm range)
			
Containing nano-metals or oxides			
Associated with another AI (μm range)		Alone (1–30 nm)	
			

Silva et al. 2011; Song et al. 2012; Stadler et al. 2010, 2012; Xiang et al. 2013; Yin et al. 2012).

Conclusions

Nanotechnology and nanoscience has attracted the scientists and researchers working on different areas. Nanoformulations are expected to have strong impacts on the fate of active ingredient and to find new ingredients whose environmental fate is not well studied. The present status cannot permit a fair evaluation of the merits and demerits that come from exploration of nanopesticides. There is a need to use the advance instrumentation to detect, characterise and quantify the active ingredient and adjuvants emanating from nanoformulations. There is urgency of thorough risk assessments channel for the nanopesticides. Further there is a need to carry out the research on environmental fate as well to study it under different conditions so risk evaluation of nanoparticles can be done.

References

Abreu FOMS, Oliveira EF, Paula HCB, de Paula RCM (2012) Chitosan/cashew gum nanogels for essential oil encapsulation. Carbohydr Polym 89(4):1277–1282

Adak T, Kumar J, Shakil NA, Walia S (2012) Development of controlled release formulations of imidacloprid employing novel nano-ranged amphiphilic polymers. J Environ Sci Health B 47(3):217–225

Ajeet K, Prashant S, Amit S, Arnab D, Ramesh C, Subho M (2008) Nano-sized copper as an efficient catalyst for one pot three component synthesis of thiazolidine-2, 4-dione derivatives. Catal Commun 10:17–22

Anjali CH, Sharma Y, Mukherjee A, Chandrasekaran N (2012) Neem oil (Azadirachta indica) nanoemulsion—a potent larvicidal agent against Culex quinquefasciatus. Pest Manag Sci 68(2):158–163

Balaji AP, Mishra P, Suresh Kumar RS, Ashu A, Margulis K, Magdassi S, Mukherjee A, Chandrasekaran N (2015) The environmentally benign form of pesticide in hydrodispersive nanometric form with improvedefficacyagainstadultmosquitoesatlowexposureconcentrations. Bull Environ Contam Toxicol 95:734–739

Balaji AP, Sastry TP, Manigandan S, Mukherjee A, Chandrasekaran N (2017) Environmental benignity of a pesticide in soft colloidal hydrodispersive nanometric form with improved toxic precision towards the target organisms than non-target organisms. Sci Total Environ 579:190–201

Belz J, Castilla-Ojo N, Sridhar S, Kumar R (2017) Radiosensitizing silica nanoparticles encapsulating docetaxel for treatment of prostate cancer. Methods Mol Biol 1530:403–409

Bhagat D, Samanta SK, Bhattacharya S (2013) Efficient management of fruit pests by pheromone nanogels. Sci Rep 3:1294

Brunel FE, Gueddari NE, Moerschbacher BM (2013) Complexation of copper(II) with chitosan nanogels: toward control of microbial growth. Carbohydr Polym 92(2):1348–1356

Buonasera K, D'Orazio G, Fanali S, Dugo P, Mondello L (2009) Separation of organophosphorus pesticides by using nano-liquid chromatography. J Chromatogr A 1216:3970–3976

Chaichi MJ, Alijanpour SO (2014) Chitosan-induced Au/Ag nanoalloy dispersed in IL and application in fabricating an ultrasensitive glucose biosensor based on luminol-H_2O_2-Cu^{2+}/IL chemiluminescence system. J Photochem Photobiol B 140:41–48

Choudhary SR, Pradhan S, Goswami A (2012) Preparation and characterisation of acephate nano-encapsulated complex. Nanosci Methods 1(1):9–15

Debnath N, Das S, Seth D, Chandra R, Bhattacharya SC, Goswami A (2011) Entomotoxic effect of silica nanoparticles against *Sitophilus oryzae* (L.) J Pest Sci 84(1):99–105

Debnath N, Mitra S, Das S, Goswami A (2012) Synthesis of surface functionalized silica nanoparticles and their use as entomotoxic nanocides. Powder Technol 221:252–256

Diaz-Blancas V, Medina DI, Padilla-Ortega E, Bortolini-Zavala R, Olvera-Romero M, LunaBarcenas G (2016) Nanoemulsion formulations of fungicide tebuconazole for agricultural applications. Molecules 21:1271

Ebrahimi M, Es'haghi Z, Samadi F, Hosseini MS (2011) Ionic liquid mediated solgel sorbents for hollow fiber solidphase microextraction of pesticide residues in water and hair samples. J Chromatogr A 1218:8313–8321

Efremenko EN, Lyagin IV, Klyachko NL, Bronich T, Zavyalova NV, Jiang Y, Kabanov AV (2017) A simple and highly effective catalytic nanozyme scavenger for organophosphorus neurotoxins. J Control Release 247:175–181

Ellison PA, Chen F, Goel S, Barnhart TE, Nickles RJ, DeJesus OT, Cai W (2017) Intrinsic and stable conjugation of thiolated mesoporous silica nanoparticles with radioarsenic. ACS Appl Mater Interfaces 9(8):6772–6781. https://doi.org/10.1021/acsami.6b14049

Ghormade V, Deshpande MV, Paknikar KM (2011) Perspectives for nano-biotechnology enabled protection and nutrition of plants. Biotechnol Adv 29:792–803

Ginet N, Pardoux R, Adryanczyk G, Garcia D, Brutesco C, Pignol D (2011) Single-step production of a recyclable nanobiocatalyst for organophosphate pesticides biodegradation using functionalized bacterial magnetosomes. PLoS One 6:e21442

Ginjupalli GK, Baldwin WS (2013) The time- and age-dependent effects of the juvenile hormone analog pesticide, pyriproxyfen on Daphnia magna reproduction. Chemosphere 92:1260–1266

Grillo R, dos Santos NZ, Maruyama CR, Rosa AH, de Lima R, Fraceto LF (2012) Poly(epsilon-caprolactone) nanocapsules as carrier systems for herbicides: physico-chemical characterization and genotoxicity evaluation. J Hazard Mater 231:1–9

Grillo R, Abhilash PC, Fraceto LF (2016) Nanotechnology applied to bio-encapsulation of pesticides. J Nanosci Nanotechnol 16:1231–1234

Hake H, Ben-Zur R, Schechter I, Anders A (2007) Fast optical assessment of pesticide coverage on plants. Anal Chim Acta 596:1–8

Herrero MA, Guerra J, Myers VS, Gomez MV, Crooks RM, Prato M (2010) Gold dendrimer encapsulated nanoparticles as labeling agents for multiwalled carbon nanotubes. ACS Nano 4:905–912

Hu X, Zheng P, Meng G, Huang Q, Zhu C, Han F, Huang Z, Li Z, Wang Z, Wu N (2016) An ordered array of hierarchical spheres for surface-enhanced Raman scattering detection of traces of pesticide. Nanotechnology 27:384001

Hubert J, Stejskal V, Munzbergova Z, Hajslova J, Arthur FH (2007) Toxicity and efficacy of selected pesticides and new acaricides to stored product mites (Acari: Acaridida). Exp Appl Acarol 42:283–290

Hummel HE, Langner SS, Eisinger MT (2013) Pheromone dispensers, including organic polymer fibers, described in the crop protection literature: comparison of their innovation potential. Commun Agric Appl Biol Sci 78:233–252

Hwang IC, Kim TH, Bang SH, Kim KS, Kwon HR, Seo MJ et al (2011) Insecticidal effect of controlled release formulations of etofenprox based on nano-bio technique. J Fac Agric Kyushu Univ 56(1):33–40

Itoh H, Naka K, Chujo Y (2004) Synthesis of gold nanoparticles modified with ionic liquid based on the imidazolium cation. J Am Chem Soc 126:3026–3027

Jerobin J, Sureshkumar RS, Anjali CH, Mukherjee A, Chandrasekaran N (2012) Biodegradable polymer based encapsulation of neem oil nanoemulsion for controlled release of Aza-A. Carbohydr Polym 90(4):1750–1756

Jiang LC, Basri M, Omar D, Rahman MBA, Salleh AB, Rahman RNZRA et al (2012) Green nano-emulsion intervention for water-soluble glyphosate isopropylamine (IPA) formulations in controlling Eleusine indica (E. indica). Pestic Biochem Physiol 102(1):19–29

Jokar M, Safaralizadeh MH, Hadizadeh F, Rahmani F, Kalani MR (2016) Design and evaluation of an apta-nano-sensor to detect Acetamiprid in vitro and in silico. J Biomol Struct Dyn 34:2505–2517

Kah M (2015) Nanopesticides and nanofertilizers: emerging contaminants or opportunities for risk mitigation? Front Chem 3:64

Kah M, Hofmann T (2014) Nanopesticide research: current trends and future priorities. Environ Int 63:224–235

Kah M, Machinski P, Koerner P, Tiede K, Grillo R, Fraceto LF, Hofmann T (2014) Analysing the fate of nanopesticides in soil and the applicability of regulatory protocols using a polymer-based nanoformulation of atrazine. Environ Sci Pollut Res Int 21:11699–11707

Kah M, Weniger AK, Hofmann T (2016) Impacts of (nano) formulations on the fate of an insecticide in soil and consequences for environmental exposure assessment. Environ Sci Technol 50(20):10960–10967

Kalayou S, Granum C, Berntsen HF, Groseth PK, Verhaegen S, Connolly L, Brandt I, de Souza GA, Ropstad E (2016) Label-free based quantitative proteomics analysis of primary neonatal porcine Leydig cells exposed to the persistent contaminant 3-methylsulfonyl-DDE. J Proteome 137:68–82

Kamlesh K, Prashant S, Gopal M (2012a) Synthesis and characterization of rosiglitazone loaded magnetic nanopolymer. Int J Green Nanotechnol 4:339–344

Kamlesh K, Prashant S, Gopal M (2012b) A facile one pot synthesis of collagen protected gold nanoparticles using Na–malanodialdehyde. Mater Lett 79:199–201

Kamlesh K, Prashant S, Gopal KM (2014) Ionic liquid stabilized Metal NPs & their role a potent catalyst. In: Tiwari A (ed) Advanced Materials. WILEY-Scrivener Publisher, Chapter 14, 549–564

Kamlesh K, Prashant S, Chandra M (2017) Metal (Au, Ag & Cu) NPs in ionic liquid: Potential Catalytic system for organic reactions. J Nanomed Nanotechnol 8(6):1–11

Kang MA, Seo MJ, Hwang IC, Jang C, Park HJ, YM Y et al (2012) Insecticidal activity and feeding behavior of the green peach aphid, Myzus persicae, after treatment with nano types of pyrifluquinazon. J Asia Pac Entomol 15(4):533–541

Kaushik P, Shakil NA, Kumar J, Singh MK, Yadav SK (2013) Development of controlled release formulations of thiram employing amphiphilic polymers and their bioefficacy evaluation in seed quality enhancement studies. J Environ Sci Health B 48(8):677–685

Khandelwal N, Barbole RS, Banerjee SS, Chate GP, Biradar AV, Khandare JJ, Giri AP (2016) Budding trends in integrated pest management using advanced micro- and nano-materials: challenges and perspectives. J Environ Manag 184:157–169

Khodadoust S, Ghaedi M, Hadjmohammadi MR (2013) Dispersive nano solid material-ultrasound assisted microextraction as a novel method for extraction and determination of bendiocarb and promecarb: response surface methodology. Talanta 116:637–646

Kim SW, Jung JH, Lamasal K, Kim YS, Min JS, Lee YS (2012) Antifungal effects of silver nanoparticles (AgNPs) against various plant pathogenic fungi. Mycobiology 40(1):53–58

Kookana RS, Boxall AB, Reeves PT, Ashauer R, Beulke S, Chaudhry Q, Cornelis G, Fernandes TF, Gan J, Kah M, Lynch I, Ranville J, Sinclair C, Spurgeon D, Tiede K, Van den Brink PJ (2016) Nanopesticides: guiding principles for regulatory evaluation of environmental risks. J Agric Food Chem 62:4227–4240

Kumar RSS, Shiny PJ, Anjali CH, Jerobin J, Goshen KM, Magdassi S et al (2013) Distinctive effects of nano-sized permethrin in the environment. Environ Sci Pollut Res 20(4):2593–2602

Kumar PM, Murugan K, Madhiyazhagan P, Kovendan K, Amerasan D, Chandramohan B, Dinesh D, Suresh U, Nicoletti M, Alsalhi MS, Devanesan S, Wei H, Kalimuthu K, Hwang JS, Lo Iacono A, Benelli G (2016) Biosynthesis, characterization, and acute toxicity of Berberis tinctoria-fabricated silver nanoparticles against the Asian tiger mosquito, aedes albopictus, and the mosquito predators toxorhynchites splendens and mesocyclops thermocyclopoides. Parasitol Res 115:751–759

Laranjeira M, Shirosaki Y, Yoshimatsu Yasutomi S, Miyazaki T, Monteiro FJ (2017) Enhanced biosafety of silica coated gadolinium based nanoparticles. J Mater Sci Mater Med 28:46

Larguinho M, Baptista PV (2011) Gold and silver nanoparticles for clinical diagnostics from genomics to proteomics. J Proteome 75:2811–2823

Loha KM, Shakil NA, Kumar J, Singh MK, Adak T, Jain S (2011) Release kinetics of β-cyfluthrin from its encapsulated formulations in water. J Environ Sci Health B 46(3):201–206

Loha KM, Shakil NA, Kumar J, Singh M, Srivastava C (2012) Bio-efficacy evaluation of nanoformulations of β-cyfluthrin against Callosobruchus maculatus (Coleoptera: Bruchidae). J Environ Sci Health B 47(7):687–691

Mingming A, Yuncong Z, Shun H, Deguang L, Pingliang L, Jianqiang L et al (2013) Preparation and characterization of 1-naphthylacetic acid-silica conjugated nanospheres for enhancement of controlled-released performance. Nanotechnology 24(3):035601–035608

Mirabelli MF, Wolf JC, Zenobi R (2016) Pesticide analysis at ppt concentration levels: coupling nano-liquid chromatography with dielectric barrier discharge ionization-mass spectrometry. Anal Bioanal Chem 408:3425–3434

Mishra P, Balaji AP, Swathy JS, Paari AL, Kezhiah M, Tyagi BK, Mukherjee A, Chandrasekaran N (2016) Stability assessment of hydro dispersive nanometric permethrin and its biosafety study towards the beneficial bacterial isolate from paddy rhizome. Environ Sci Pollut Res Int 23:24970–24982

Momeni S, Nabipour I (2015) A simple green synthesis of palladium nanoparticles with sargassum alga and their electrocatalytic activities towards hydrogen peroxide. Appl Biochem Biotechnol 176:1937–1949

Mondal KK, Mani C (2012) Investigation of the antibacterial properties of nanocopper against Xanthomonas axonopodis pv. punicae, the incitant of pomegranate bacterial blight. Ann Microbiol 62(2):889–893

Narayanan N, Gupta S, Gajbhiye VT, Manjaiah KM (2017) Chemosphere optimization of isotherm models for pesticide sorption on biopolymer-nanoclay composite by error analysis. Parasitol Res 173:502–511

Niikura K, Matsunaga T, Suzuki T, Kobayashi S, Yamaguchi H, Orba Y, Kawaguchi A, Hasegawa H, Kajino K, Ninomiya T, Ijiro K, Sawa H (2013) Gold nanoparticles as a vaccine platform: influence of size and shape on immunological responses in vitro and in vivo. ACS Nano 7:3926–3938

Nuruzzaman M, Rahman MM, Liu Y, Naidu R (2016) Nanoencapsulation, nano-guard for pesticides: a new window for safe application. J Agric Food Chem 64:1447–1483

Paret ML, Palmateer AJ, Knox GW (2013a) Evaluation of a light-activated nanoparticle formulation of titanium dioxide with zinc for management of bacterial leaf spot on rosa ‘Noare. Hortscience 48(2):189–192

Paret ML, Vallad GE, Averett DR, Jones JB, Olson SM (2013b) Photocatalysis: effect of light-activated nanoscale formulations of TiO_2 on Xanthomonas perforans and control of bacterial spot of tomato. Phytopathology 103(3):228–236

Periasamy AP, Umasankar Y, Chen SM (2009) Nanomaterials - acetylcholinesterase enzyme matrices for organophosphorus pesticides electrochemical sensors: a review. Sensors (Basel) 9:4034–4055

Petosa AR, Rajput F, Selvam O, Ohl C, Tufenkji N (2016) Assessing the transport potential of polymeric nanocapsules developed for crop protection. Water Res 111:10–17

Pradeep K, Prashant S, Kamlesh K, Subho M, Ramesh C (2011) A green approach for the synthesis of gold nanotriangles using aqueous leaf extract of Callistemon viminalis. Mater Lett 65:595–597

Prashant S (2013) A simple, rapid, and green synthesis of capped gold nanospheres and nanorods using aqueous extract of azolla. Int J Green Nano 1:1–5

Prashant S, Sunil K, Anju K, Rashmi K, Ramesh C (2008a) A novel route for the synthesis of indium nanoparticles in ionic liquid. Mater Lett 62:4164–4166

Prashant S, Anju K, Rashmi K, Ramesh C (2008b) Copper nanoparticles in ionic liquid: an easy and efficient catalyst for the coupling of thiazolidine-2,4-dione, aromatic aldehyde and ammonium acetate. Catal Commun 9:1618–1623

Prashant S, Anju K, Rashmi K, Ramesh C (2008c) Copper nanoparticles in an ionic liquid: an efficient catalyst for the synthesis of bis-(4-hydroxy-2-oxothiazolyl)methanes. Tetrahedron Lett 49:727–730

Prashant S, Kamlesh K, Anju K, Rashmi K, Ramesh C (2009a) Synthesis and characterization of silver and gold nanoparticles in ionic liquid. Spectrochim Acta A Mol Biomol Spectrosc 73:218–220

Prashant S, Kamlesh K, Anju K, Rashmi K, Ramesh C (2009b) Copper nanoparticles in ionic liquid: an easy and efficient catalyst for selective Cara-Michael addition reaction. Catal Lett 127:119–125

Prashant S, Kamlesh K, Anju K, Rashmi K, Ramesh C (2009c) Cu nanoparticles in ionic liquid: an easy and efficient catalyst for addition–elimination reaction between active methylene compounds and imines in an ionic liquid. Catal Lett 130:648–654

Prashant S, Pradeep K, Kamlesh K, Pankaj S, Subho M, Ramesh C (2011) A rapid and simple route for the synthesis of lead and palladium nanoparticles in tetrazolium based ionic liquid. Spectrochim Acta A Mol Biomol Spectrosc 78:909–912

Prashant S, Kamlesh K, Vijay V, Gopal M, Durgesh K, Vaishali S, Ramesh C (2017) Metal NPs (Au, Ag, and Cu): Synthesis, stabilization, and their role in green chemistry and drug delivery. In: Singh R, Kumar S (eds) Green technologies and environmental sustainability. Springer, Cham. Chapter 14, 309–338

Qian K, Shi T, Tang T, Zhang S, Liu X, Cao Y (2011) Preparation and characterization of nano-sized calcium carbonate as controlled release pesticide carrier for validamycin against Rhizoctonia solani. Microchim Acta 173(1–2):51–57

Qing S, Yongli S, Yuehong Z, Ting Z, Haoyan S (2013) Pesticide-conjugated polyacrylate nanoparticles: novel opportunities for improving the photostability of emamectin benzoate. Polym Adv Technol 24(2):137–143

Sarkar DJ, Kumar J, Shakil NA, Walia S (2012) Release kinetics of controlled release formulations of thiamethoxam employing nano-ranged amphiphilic PEG and diacid based block polymers in soil. J Environ Sci Health A Tox Hazard Subst Environ Eng 47(11):1701–1712

Scheeren CW, Machado G, Teixeira SR, Morais J, Domingos JB, Dupont J (2006) Synthesis and characterization of Pt(0) nanoparticles in imidazolium ionic liquids. J Phys Chem B 110:13011–13020

Silva MS, Cosenza DS, Grillo R, Melo NFS, Tonello PS, Oliveira LC et al (2011) Paraquat-loaded alginate/chitosan nanoparticles: preparation, characterization and soil sorption studies. J Hazard Mater 190(1–3):366–374

Song MR, Cui SM, Gao F, Liu YR, Fan CL, Lei TQ et al (2012) Dispersible silica nanoparticles as carrier for enhanced bioactivity of chlorfenapyr. J Pestic Sci 37(3):258–260

Stadler T, Buteler M, Weaver DK (2010) Novel use of nanostructured alumina as an insecticide. Pest Manag Sci 66(6):577–579

Stadler T, Buteler M, Weaver DK, Sofe S (2012) Comparative toxicity of nano structured alumina and a commercial inert dust for Sitophilus oryzae (L.) and Rhyzopertha dominica (F.) at varying ambient humidity levels. J Stored Prod Res 48:81–90

Wang L, Su M, Zhao X, Hong J, Yu X, Xu B, Sheng L, Liu D, Shen W, Li B, Hong F (2015) Nanoparticulate TiO_2 protection of midgut damage in the silkworm (Bombyx mori) following phoxim exposure. Arch Environ Contam Toxicol 68:534–542

Wang Y, Sun C, Zhao X, Cui B, Zeng Z, Wang A, Liu G, Cui H (2016) The application of nano-TiO_2 photo semiconductors in agriculture. Nanoscale Res Lett 11:529

Wilcoxon J (2009) Optical absorption properties of dispersed gold and silver alloy nanoparticles. J Phys Chem B 113:2647–2656

Xiang C, Taylor AG, Hinestroza JP, Frey MW (2013) Controlled release of nonionic compounds from poly(lactic acid)/cellulose nanocrystal nanocomposite fibers. J Appl Polym Sci 127(1):79–86

Xiang GQ, Ren Y, Xia Y, Mao W, Fan C, Guo SY, Wang PP, Yang DH, He L, Jiang X (2017) Carbon-dot-based dual-emission silica nanoparticles as a ratiometric fluorescent probe for bisphenol. Spectrochim Acta A Mol Biomol Spectrosc 177:153–157

Xie W, Su L, Donfack P, Shen A, Zhou X, Sackmann M, Materny A, Hu J (2009) Synthesis of gold nanopeanuts by citrate reduction of gold chloride on gold–silver core–shell nanoparticles. Chem Commun (Camb):5263–5265

Xu X, Li Y, Gong Y, Zhang P, Li H, Wang Y (2012) Synthesis of palladium nanoparticles supported on mesoporous N-doped carbon and their catalytic ability for biofuel upgrade. J Am Chem Soc 134:16987–16990

Xu Y, Wang Y, Li K, Niu M, Zeng Y, Jiang J, Jin Z (2013) Rh nanoparticle catalyzed hydroaminomethylation of 1-octene in thermoregulated ionic liquid and organic biphasic system. J Nanosci Nanotechnol 13:5048–5052

Yang G, Zhao F, Zeng B (2014) Facile fabrication of a novel anisotropic gold nanoparticle-Chitosan-ionic liquid/Graphene modified electrode for the determination of theophylline and caffeine. Talanta 127:116–122

Yao HB, Mao LB, Yan YX, Cong HP, Lei X, SH Y (2012) Gold nanoparticle functionalized artificial nacre: facile in situ growth of nanoparticles on montmorillonite nanosheets, self-assembly, and their multiple properties. ACS Nano 6:8250–8260

Yao J, Sun N, Wang J, Xie Y, Deng C, Zhang X (2017) Rapid synthesis of titanium(IV)-immobilized magnetic mesoporous silica nanoparticles for endogenous phosphopeptides enrichment. Proteomics 17(8):1600320. https://doi.org/10.1002/pmic.201600320

Yin YH, Guo QM, Han Y, Wang LJ, Wan SQ (2012) Isolation and characterization of NBSLRR class resistance homologous gene from wheat. J Integr Agric 1(7):1151–1158

Yu B, Zeng J, Gong L, Zhang M, Zhang L, Chen X (2007) Investigation of the photocatalytic degradation of organochlorine pesticides on a nano-TiO_2 coated film. Talanta 72:1667–1674

Zapp E, Westphal E, Gallardo H, de Souza B, Cruz Vieira I (2014) Liquid crystal and gold nanoparticles applied to electrochemical immunosensor for cardiac biomarker. Biosens Bioelectron 59:127–133

Zhang H, Cui H (2009) Synthesis and characterization of functionalized ionic liquid-stabilized metal (gold and platinum) nanoparticles and metal nanoparticle/carbon nanotube hybrids. Langmuir 25:2604–2612

Zhang HJ, Zheng HZ, Long YJ, Xiao GF, Zhang LY, Wang QL, Gao M, Bai WJ (2012) Gold nanoparticles as a label-free probe for the detection of amyloidogenic protein. Talanta 89:401–406

Zheng Q, Yu Y, Fan K, Ji F, Wu J, Ying Y (2016) A nano-silver enzyme electrode for organophosphorus pesticide detection. Anal Bioanal Chem 408:5819–5827

Zheng Y, Fahrenholtz C, Hackett C, Ding S, Day C, Dhall R, Marrs G, Gross M, Singh R, Bierbach U (2017) Large-pore functionalized mesoporous silica nanoparticles as drug delivery vector for a highly cytotoxic hybrid platinum–acridine anticancer agent. Chemistry 23(14):3386–3397. https://doi.org/10.1002/chem.201604868